Characterisation of *in vitro* excretory-secretory components of the ovine intestinal nematode, *Trichostrongylus vitrinus*

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Dedication

To Mum and Dad
for all their love, encouragement and patience
throughout my academic career.

Abstract

Trichostrongylus vitrinus is one of the principal causative nematodes of ovine parasitic gastro-enteritis within Scotland and infests the proximal small intestine of the sheep. At present, control is achieved mainly by the administration of anthelmintic drugs, but with the increasing emergence of anthelmintic resistance, much research is now centred on vaccine development. Recent evidence has suggested that the excretory-secretory components (ES) from parasitic nematodes may be an important source of host-protective antigens. The overall aim of the present study was to characterise the nature and properties of *T. vitrinus* ES components.

The initial part of the work involved the partial characterisation of two of the enzymes, acetylcholinesterase (AChE) and proteinases, excreted and secreted during the *in vitro* culturing of adult *T. vitrinus*. These enzymes, were defined on the basis of their substrate specificity, molecular size, pH optima and inhibitor sensitivity.

Attempts were also made to isolate complementary DNA (cDNA) fragments encoding AChE from an adult *T. vitrinus* cDNA pool, using the polymerase chain reaction (PCR) and degenerate oligonucleotide primers directed towards highly conserved regions of AChEs that had previously been identified by comparison of known polypeptide sequences from a number of higher eukaryotic organisms. No adult *T. vitrinus* cDNA fragments encoding AChE were amplified, suggesting that *T. vitrinus* AChE(s) is/are distinctly different to AChEs from higher eukaryotes, at least at the level of nucleic acid sequence.

Subsequent research focused on the molecular characterisation of adult *T. vitrinus* ES. Immunoscreening of an adult *T. vitrinus* cDNA lambda gt11 library with antiserum raised against adult *T. vitrinus* ES, resulted in the isolation of ten immunopositive clones. Their inserts were sequenced and the results were analysed using computer databases. Three of the clones were identified as harbouring inserts that encoded proteins that shared significant homology to myosin heavy chain, vitellogenin and serine proteinase inhibitor (serpin) respectively. The other seven clones contained inserts that showed no significant homology to any of the sequences present in the computer databases.

Serpins have not been previously identified in the ES of parasitic nematodes and may facilitate the evasion of host anti-parasite immune responses. Northern blot analysis, using the serpin cDNA as a probe, revealed a messenger RNA (mRNA) transcript of approximately 1.2 kb. This is in accord with the predicted size required to encode other known serpins. Using PCR and specific oligonucleotide primers, a full length cDNA encoding this serpin was sought. This procedure demonstrated the production of at least three very closely related mRNA transcripts, as judged by sequence comparison.

The potential importance of putative ES serpins for parasite survival within the host is discussed. Such serpins may provide novel targets for anthelmintic drug design or, as antigens for vaccination.

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Contents

Abbreviations

AChE	acetylcholinesterase
α_1-AT	α_1-antitrypsin
ATCI	acetylthiocholine iodide
ATP	adenosine-5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin (fraction V)
BuChE	butyrylcholinesterase
BZ	benzimidazole
°C	degrees Celsius
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal alkaline phosphatase
cm	centimetre(s)
$CuSO_4$	copper sulphate
DAB	diaminobenzidine tetra-hydrochloride
d(d)ATP	2'(3'-di) deoxyadenosine-5'-triphosphate
d(d)CTP	2'(3'-di) deoxycytidine-5'-triphosphate
d(d)GTP	2'(3'-di) deoxyguanosine-5'-triphosphate
d(d)TTP	2'(3'-di) deoxythymidine-5'-triphosphate
d(d)NTP	2'(3'-di) deoxynucleotide-5'-triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNTB	5'5'-dithiobi-2-nitrobenzoic acid
E64	trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane
ECL	enhanced chemiluminescence
EM	electron microsope
ES	excretory-secretory components
FEC	faecal egg count
g	gravitational centrifugal force
GI	gastro-intestine
GL	globule leucocytes
gm	gram(mes)
GST	glutathione-S-transferase
H^+	hydrogen ion(s)
HCl	hydrochloric acid
HPTI	human placental thrombin inhibitor
hr	hour(s)
HRP	horse radish peroxidase
5-HT	5-hydroxytryptamine
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPTG	isopropylthio-β-D-galactoside
IU	international units
Kb	kilobase(s)
KBP	kallikrein-binding protein

KBq	kilobecqueral(s)
kDA	kilodalton(s)
kg	kilogram(mes)
KH_2PO_4	potassium dihydrogen orthophosphate
l	litre(s)
L1	first stage larvae
L2	second stage larvae
L3	infective third stage larvae
L4	fourth stage larvae
L5	fifth stage larvae
λ	lambda phage
LEI	leucocyte elastase inhibitor
M	molar
mA	millampere(s)
MBq	megabequerel(s)
MCS	multiple cloning site
mg	milligram(mes)
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minute(s)
MHC	major histocompatability complex
ml	millilitre(s)
μCi	microcurie(s)
μg	microgram(mes)
μm	micrometre(s)
μM	micromolar
MOPS	3-(morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
Na_2EDTA	disodium ethylenedianinetetra-acetic acid
$NaHCO_3$	sodium hydrogen carbonate
Na_2HPO_4	sodium hydrogen orthophosphate
NaH_2PO_4	sodium dihydrogen phosphate
NaOH	sodium hydroxide
nm	nanometre(s)
O_2	oxygen
OD	optical density
ORF	open reading frame
%	percentage
^{32}P	β-emitting isotope of phosphorus
PAGE	polyacrylamide gel electrophoresis
PAI-2	plasminogen activator inhibitor 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit(s)
PGE	parasitic gastro-enteritis
pH	$-\log_{10}$ (hydrogen ion concentration)

p.i.	post infection
PmsF	phenylmethylsulphonyl fluoride
polyA+	polyadenylated
psi	pounds per square inch
RE	rapid expulsion
RNA	ribonucleic acid
rpm	revolutions per minute
^{35}S	β-emitting isotope of sulphur
sAChE	secretory AChE
SCC	saline sodium citrate
SDS	sodium dodecyl sulphate
serpin	serine proteinase inhibitor
SL1	*trans*-spliced leader 1
SL2	*trans*-spliced leader 2
SMCP	sheep mast cell proteinase
SOD	superoxide dismutase
SPI3	serine proteinase inhibitor 3
spp.	species
TAE	tris-acetate/Na_2EDTA buffer
TBE	tris-borate/Na_2EDTA buffer
TBS	tris buffered saline
TBST	TBS with Tween
TBq	terabecquerel(s)
TCA	tetrachloroacetic acid
TE	tris/Na_2EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
T_H	T helper cell
Tris	tris (hydroxymethyl) amino methane
Triton-X-100	octylphenoxypolyethoxyethanol
U	unit(s)
UK	United Kingdom
UV	ultra violet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XL3	exsheathed third stage larvae
W	watt(s)
w/v	weight per volume
V	volt(s)
vs.	versus
v/v	volume per volume
v/v/v	volume per volume per volume

One letter symbols for nucleotides:

Nucleotide	One-letter symbol	Nucleotide	One-letter symbol
adenosine	A	thymidine	T
cytidine	C	inosine	I
guanosine	G	A, C, G or T	N

Three and one letter symbols for the amino acids:

Amino acid	Three-letter symbol	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valanine	Val	V
Unknown		X

Chapter one

Introduction

1.1 GENERAL INTRODUCTION

Parasitic gastro-enteritis (PGE) is a major cause of production loss in sheep, particularly in lambs during their first grazing season. Such losses result from impaired growth, poor carcass and fleece quality and also, the cost associated with current control procedures. These together result in considerable economic loss to the sheep industry world-wide (Brunsden, 1980) and production loss in the UK alone is estimated at £50 million/year (Dr. F. Jackson, Moredun Research Institute, Edinburgh, UK, personal communication).

The disease, PGE, is caused by infestation with trichostrongyle worms that parasitise the ovine alimentary canal. The principal causative nematodes are *Ostertagia circumcincta* (recently reclassified as *Teladorsagia circumcincta*), *Haemonchus contortus* and *Trichostrongylus axei* which parasitise the abomasal glands and also, *Trichostrongylus vitrinus*, *Trichostrongylus colubriformis* and *Nematodirus battus* which inhabit the proximal small intestine. In Scotland, during the summer, *O. circumcincta* predominates, while during the winter months, *T. vitrinus* is the most prevalent (Reid and Armour, 1975).

At present, control of these parasites is achieved by the use of anthelmintic drugs and grazing management. However, the development of anthelmintic resistance in nematode populations and increasing consumer concern over the presence of chemical residues in meat and the environment, has hastened the search for alternative approaches for the control of PGE nematodes. The development of an immunologically based control procedure should be feasible as, after a period of time, sheep continually exposed to infection do gain immunity to subsequent challenge (Waller and Thomas, 1981).

The work carried out in this thesis focuses on *T. vitrinus*, an ovine intestinal nematode of particular importance in Scotland and Northern England (Reid and Armour, 1975). However, most research on *Trichostrongylus* spp. to date has been on the closely related *T. colubriformis*, which is more endemic in other temperate regions of the world, such as Australia. The following literature review will concentrate mainly on past research on *Trichostrongylus* spp., and also, will

encompass a wider range of gastro-intestinal (GI) nematodes and other helminths in a more general discussion on approaches to the future control of parasites.

1.2 PHYLOGENY AND STRUCTURE

The phylogeny of *Trichostrongylus* spp. is summarised in figure 1.1 (Soulsby, 1968; Gibbons and Khalil, 1982). Nematoda are frequently termed "roundworms" because of their circular cross-section, are elongated in shape, unsegmented and usually are tapering at the extremities. *Trichostrongylus* spp. are small (<1 cm), slender, pale reddish-brown worms without a specially developed head (Soulsby, 1968).

Figure 1.1 Phylogeny of *Trichostrongylus* spp.

Kingdom: **Animalia**

↓

Phylum: **Nemathelminths**

↓

Class: **Nematoda**

(free-living and parasitic nematodes)

↓

Order: **Strongyloidea**

(many animal nematodes)

↓

Family: **Trichostrongylidae**

(several ovine GI nematodes)

↓

Genus: ***Trichostrongylus***

↓

Species: e.g. ***T. axei, T. colubriformis, T. vitrinus***

1.3 LIFE CYCLE OF *TRICHOSTRONGYLUS* SPP.

The life cycle of *Trichostrongylus* spp. is direct, consists of two stages and is very similar to the life cycles of the majority of Trichostrongylids (figure 1.2).

Non-parasitic, free-living stage

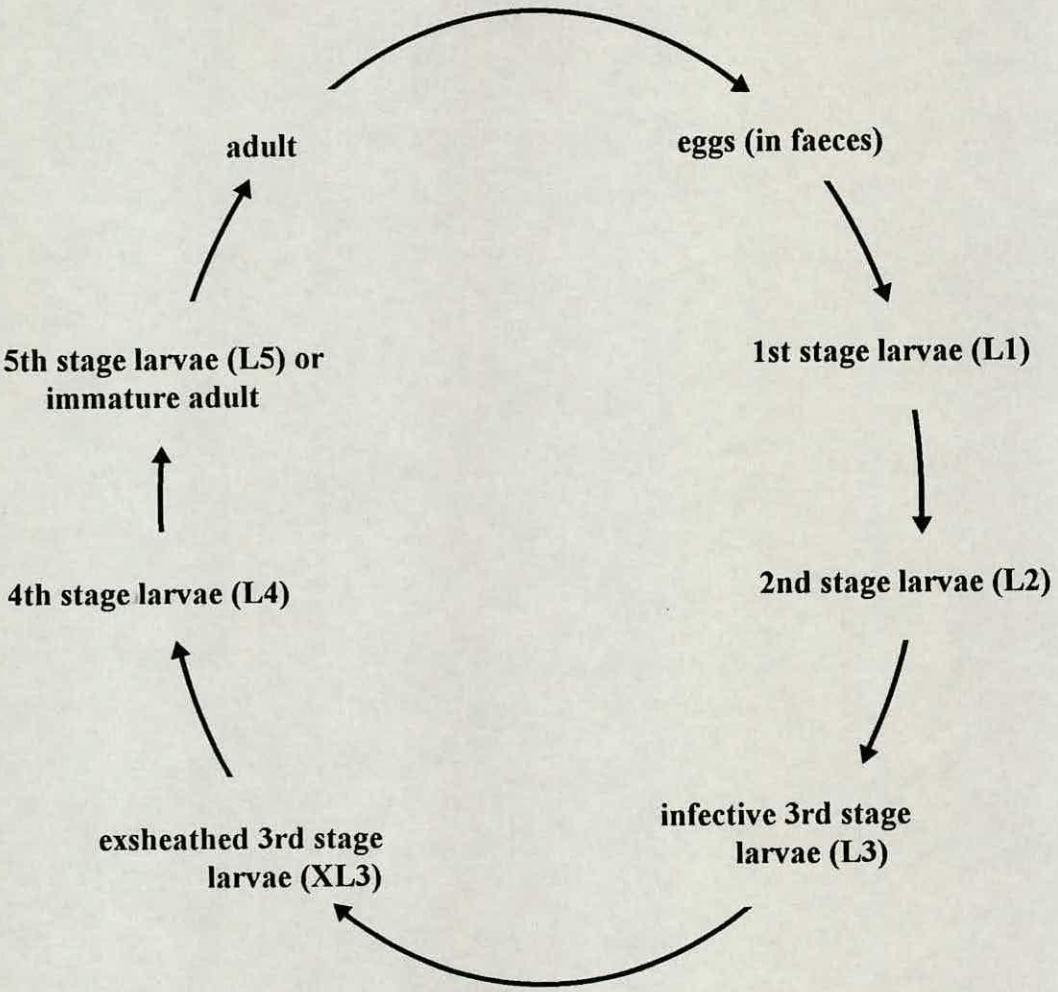
Eggs are passed onto the pasture via the faeces of the infected host, by which time they have usually divided into 16 - 32 cells (Ross and Gordon, 1936). Under optimal conditions, the embryonated eggs develop into first stage (L1) larvae within 20 - 24 hours (Soulsby, 1968). After hatching, the L1 feeds on faecal microflora until, after a further 12 hours, it moults (ecdysis) to become a second stage (L2) larvae. A second ecdysis occurs 20 - 40 hours later, during which the L2 cuticle is not cast off but is retained as a loose protective sheath around the third stage (L3) larvae. Hatching and development of the free-living stages is dependent on the temperature and humidity of the microenvironment. Also, the larvae have a predominantly aerobic metabolism and are sensitive to lack of O_2 (Gibbs, 1982). *T. vitrinus* eggs are able to hatch and develop to L3 at temperatures as low as 4°C, whereas *T. colubriformis* require the temperature to be above 10°C for development (Beveridge *et al.*, 1989). Ambient temperatures, therefore, can influence the prevalence of *T. vitrinus and T. colubriformis* (Rose and Small, 1984 and 1985; De Chaneet and Dunsmore, 1988). The infective L3 survives on stored food reserves and is capable of withstanding considerable desiccation. The L3 migrates from the faeces onto herbage (Rose and Small, 1985) and cannot develop further until it enters the host by ingestion with vegetation.

Parasitic stage

Once swallowed by the host, exsheathment of the L3 takes place in the abomasum. Two steps are involved in this process, first, external stimuli (H^+ and CO_2) in the abomasum cause the L3 to release an exsheathing fluid, then secondly, the enzymatic nature of this fluid removes the protective sheath (Soulsby, 1968). The composition of the exsheathing fluid is not clearly defined but in the abomasal

nematode, *H. contortus*, a zinc metallo-proteinase has been implicated in the ecdysis of L3 (Gamble, Purcell and Fetterer, 1989). Tissue infection commences when the exsheathed L3 (XL3) penetrate the small intestine at the base of the villi and develop in the subepithelial tunnels (Taylor and Pearson, 1979 a and b). After 2 - 4 days the third ecdysis, to L4, occurs and a further period of growth and differentiation precedes final ecdysis to L5, the immature adult, 15 days post-infection (p.i.). Adults reach maturity at 21 days after infection (Taylor and Pearson, 1979 a and b) and are found partially in the epitheilia, although rupture of the tunnel wall may expose parts of the worm to the intestinal lumen (Coop, Angus and Sykes, 1979; Taylor and Pearson, 1979 a, b).

Figure 1.2 Life cycle of *Trichostrongylus* spp.



1.4 PATHOGENESIS

Figure 1.3 shows a scanning electron microscopic (EM) picture of the mucosal lining of the small intestine from a worm-free lamb. In the small intestine, digestion of food is normally completed and the products are absorbed. Some fluid is also absorbed in the small intestine. The intestinal mucosa consists of a continuous sheet of epithelial cells and is folded into finger-like projections called villi. The luminal edge of the epithelium coating the villi is divided into minute microvilli which form a brush border. The combination of villi and microvilli are estimated to increase the total surface area of the small intestine by 600 fold.

Following penetration at the base of the villi, as the worm develops, it burrows through the layers of the gut (figure 1.4). This tunnelling action destroys the mucosal structure resulting in villous atrophy, 'fingerprint' lesions and crypt elongation (Taylor and Pearson, 1979 a and b; Coop, Angus and Sykes, 1979; Jackson, Angus and Coop, 1983). Figure 1.5 demonstrates the change in the intestinal mucosal topography. In an infected area on the left, erosion of the epithelial tissue has left the villi stunted, forming small rosette like structures with central depressions (Coop, Sykes and Angus, 1979). Damage to the intestine is localised to the site of worm infection and on the right of the picture, an area which is worm-free, the villi are left intact. The intestinal mucosa becomes thickened, inflamed and covered in mucin. The general appearance of the intestine from a lamb parasitised with *T. vitrinus* is of bright red flattened confluent regions very distinct from the more normal beige-coloured mucosa (figure 1.6, Coop, Sykes and Angus, 1979). Histopathological changes which occur in the intestine during infection include a cellular infiltration of mast cells, basophils, eosinophils and leukocytes (Rothwell and Dineen, 1972; Coop, Angus and Sykes, 1979) which are associated with the inflammatory response. After nine weeks of continued infection with *T. vitrinus* (2500 infective L3 given daily on five days per week), localised lesions were not observed in the lamb intestinal mucosa (3 months old at the start of the infection) suggesting partial recovery of the mucosa (Coop, Sykes and Angus, 1979; Jackson, Angus and Coop, 1983).

Activities of the brush border enzymes, such as alkaline phosphatase, and mucosal activities of the pancreatic enzymes were significantly reduced in three month old lambs, that had been continually infected with 2500 L3 *T. colubriformis* five times weekly, for 5 weeks (Jones, 1982, 1983), though 14 weeks after infection, recovery to normal enzyme levels was recorded. The activities of other enzymes, including acetylcholinesterase (AChE) became elevated (Rothwell, Ogilvie and Love, 1973; Jones, 1982 and 1983) during infection and elevations in AChE were correlated to worm burden (Jones, 1983). The recovery of the mucosal topography, as well as the restoration of normal enzyme levels, are probably related to the expulsion of the resident parasite population by mechanisms discussed in more detail below.

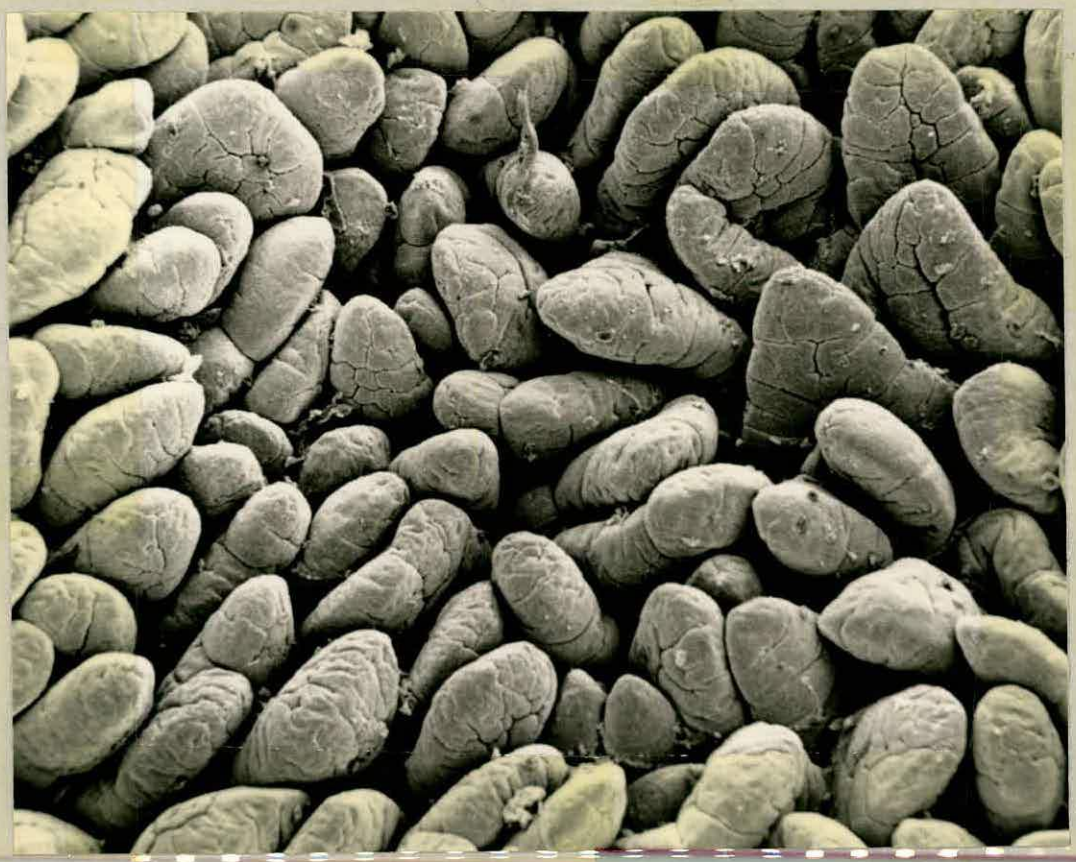
Erosion of the intestinal surface gradually increases the mucosal permeability thus disrupting the efficiency of digestion and electrolyte, mineral and water regulation, resulting in diarrhoea ('black scour') and dehydration. One of the consequences of the inflammatory response is an increase in capillary permeability and flow of blood, allowing larger molecules which are involved in immunity to migrate to the sight of infection. This, along with the physical changes in the gut due to the feeding and migratory habits of the nematode, allows leakage of blood proteins into the gut and their loss in the faeces (Yakoob, Holmes and Armour, 1983). To maintain serum protein levels and to enable damaged tissue to respond, protein and energy must be diverted away from other metabolically demanding processes, for example, milk and wool production (Sykes and Coop, 1976; Sykes, Coop and Angus, 1979).

Reduced retention of calcium and phosphorus decreases skeletal growth and mineralisation, resulting in stunted growth of the animals (Sykes, Coop and Angus, 1979; Wilson and Field, 1983). This in turn reduces the amount of muscle which accumulates (Sykes and Coop, 1976). Such carcasses also tends to contain more water (Holmes, 1986) and, in the worst cases, will be condemned at meat inspection.

Inappetance is another feature of PGE, although the reasons for this are unknown but pain and reduced gut motility may be important factors (Sykes and Coop, 1976; Symons and Hennessy, 1981).

Figure 1.3

Scanning EM (magnification x74) of the mucosal lining of the small intestine from a worm-free lamb.



The EM slide was prepared and photographed by Mr. E.W. Gray, Moredun Research Institute, Edinburgh, UK.

Figure 1.4

Scanning EM (magnification x720) of *T. vitrinus* burrowing through the mucosal lining of the the small intestine from a lamb infected with *T. vitrinus*.

The EM slide was prepared and photographed by Mr. E.W. Gray, Moredun Research Institute, Edinburgh, UK.

Figure 1.5

Scanning EM (magnification x26) of the mucosal lining of the small intestine from a lamb infected with *T. vitrinus*.

The EM slide was prepared and photographed by Mr. E.W. Gray, Moredun Research Institute, Edinburgh, UK.

Figure 1.6

Macroscopic view of the mucosal lining of the small intestine from a lamb infected with *T. vitrinus*.

1.5 ECONOMIC CONSEQUENCES

Though severe cases of GI nematodiasis may result in death, the majority of lambs suffer relatively light (sub-clinical) infections in which no obvious signs, such as diarrhoea, are evident. Continual trickle infection gradually builds host immunity to infection but productivity is still affected, with reductions in weight gain, food conversion, growth rate, fleece quality as well as adverse changes in carcass composition (Sykes and Coop, 1976; Holmes, 1986). Mild infestation may make the host more susceptible to other infections and in suckling ewes milk yield is reduced (Holmes, 1986; Jackson, Jackson and Williams, 1988). In one study, a trickle infection of *T. colubriformis* in young lambs was found to result in a 50 % reduction in weight gain (Coop, Sykes and Angus; 1976), while Steel, Symons and Jones (1980) also recorded a 53% reduction in wool growth in animals infected with the same parasite. These cumulative effects on productivity result in considerable financial loss to the sheep industry world-wide.

1.6 HOST IMMUNITY TO NATURAL INFECTION

'Self-cure'

The development of immunity in ruminants continually exposed to infection with GI nematodes is a well recorded phenomenon (e.g. Chienjina and Sewell, 1974 a and b; Waller and Thomas, 1981; Jackson, Angus and Coop, 1983). Resistance to the establishment of nematode infection occurs at about six months of age in lambs and generally precedes natural elimination ('self-cure') of the majority of worms residing in the GI tract (Waller and Thomas, 1981; Dobson, Waller and Donald, 1990 c; Barger *et al.* 1985). The sequence of events leading to 'self-cure' are rejection of incoming L3, inhibition of larval development, depression of fecundity and expulsion of adult worms (Dobson, Waller and Donald, 1990 a and c).

Chienjina and Sewell (1974 a) demonstrated that three week old lambs infected daily with 5000 *T. colubriformis* infective L3 accumulated burdens during the first twelve weeks of infection, after which, the animals developed resistance to

further challenge infection, though the ability to expel established adult worms did not develop until the lambs were six months old (Chienjina and Sewell, 1974 b). The onset of resistance to worm establishment and inhibition of parasite development appear to be two separate entities (Barger *et al.*, 1985; Seaton *et al.*, 1989). In a trickle *T. vitrinus* infection (1000 L3 daily), the first signs of immunity in lambs (six months old) after four weeks of infection was resistance to the establishment of incoming worms, with no arrested worms being evident until a later stage of challenge (Seaton *et al.*, 1989), whereas with *H. contortus* infection, the first indication of resistance was the arrested development of half the given worm burden (Barger *et al.*, 1985).

Jackson, Angus & Coop (1983) found lambs mounted a more rapid response to *T. vitrinus* than to *T. colubriformis*. Administration of 2500 *T. vitrinus* daily produced resistance in lambs after 14 weeks whereas, resistance to similar doses of *T. colubriformis* can take up to 20 weeks (unpublished data, cited in Jackson, Angus & Coop, 1983).

Several factors affect the rate of development of resistance to infection including size of larval challenge, age, reproductive status and dietary protein.

Size of larval challenge

Waller and Thomas (1981) and Dobson, Waller and Donald (1990 c) demonstrated that the development of resistance to *T. colubriformis* in lambs was dependent on worm burden. Lambs (25 weeks of age) given high *T. colubriformis* infective L3 intake (623 - 2000 daily for five days each week) started to show signs of resistance between 7 and 9 weeks after the initial infection, whereas in similar animals given smaller intakes (200 infective L3 daily) the process was delayed for five weeks (Dobson, Waller and Donald, 1990 c). This suggested that there was no rapid turnover of worms in young animals, instead worms amass in the intestine until sufficient exposure to infection stimulates the first stages of resistance (Waller and Thomas, 1981; Dobson, Waller and Donald, 1990 a and c). A similar requirement for a threshold of nematodes was observed in the development of resistance to *H. contortus* (Barger *et al.*, 1985). This contrasts with *Ostertagia ostertagia* infection in

calves (Michel, 1963, 1969) and *O. circumcincta* in lambs (Waller and Thomas, 1978) where a rapid turnover of adult worms is seen, resulting from the loss or replacement of worms at a rate depending on the current established population within the host.

Age

The first evidence that acquired resistance to *T. colubriformis* infection was age dependent was presented by Gibson, Parfitt and Everett (1970). Later work explored this further. Lambs of varying age (8 - 36 weeks) were orally dosed daily with 2000 *T. colubriformis* larvae on five days per week for 36 weeks (Gibson and Parfitt, 1972). Lambs aged 8 - 12 weeks at commencement of infection showed very high faecal egg counts (FEC) throughout the experiment and either died or were put down. In the lambs that were 16 - 28 weeks old at the beginning of the trial, FEC rose to a high plateau although the clinical signs of PGE were diminished indicating a developing immune response which increased with age. In lambs aged 32 and 36 weeks at the time of the first infection, FEC reached a low plateau and solid immunity was established as judged by final worm burdens. Similar data was presented by Dobson, Waller and Donald (1990 b). Age-related immunity has also been found with lambs infected with *H. contortus* (Manton *et al.*, 1962) and *O. circumcincta* (Smith *et al.*, 1984).

The cause of unresponsiveness is not known, although young lambs have been shown to express protective immune responses in other situations. Splenic cells from foetal lambs bind antigens (Decker and Sercaz, 1974), produce immunglobulin G (IgG) and IgM antibodies (Silverstein, Prendergast and Kramer, 1964) and have the capacity to reject skin homographs (Schinckel and Ferguson, 1953). Three reasons for unresponsiveness in young lambs have been suggested. Firstly, mature sheep (one year old) vaccinated with 1000 irradiated *H. contortus* L3 (two doses of larvae, three weeks apart) and subsequently challenged with 10000 infective L3, were found to have elevated levels of mucosal IgA and serum IgG compared to similarly treated 8 weeks old lambs (Duncan, Smith and Dargie, 1978). The development of mucosal IgA and serum IgG responses may be slow in lambs or perhaps a protective response to complex multicelluar parasites cannot be instigated until a later stage (Dobson,

Waller and Donald, 1990 b). However, Gregg *et al.* (1978) found no evidence for delayed immune maturation as three months old lambs were able to produce antibodies to one *T. colubriformis* antigen, acetylcholinesterase (AChE).

Secondly, the most striking observation noted by Gregg *et al.* (1978) was the correlation between globule leukocytes (GL) and the ability to resist challenge. GLs were far more prominent in the tissues and the small intestine of mature sheep (ten months old) than of in lambs aged three months.

Thirdly, Riffkin and Dobson (1979) observed that the lowest lymphocyte counts were in sheep which were most susceptible to infection and suggested that lambs may have a reduced lymphocyte responsiveness to parasite antigens.

Reproductive status

Lactating ewes grazing on pasture carry higher populations of the GI nematodes, *H. contortus*, *T. colubriformis* and *Ostertagia* spp. and have larger FEC compared to unmated ewes and ewes deprived of their lambs at birth (O'Sullivan and Donald, 1970). Similar experiments with *T. colubriformis* infections established that ewes lactating or in late pregnancy were immunologically suppressed compared to unmated ewes (O'Sullivan and Donald, 1973). Ewes that were <30 days post-parturition had similar worm burdens to lactating and pregnant ewes, but significantly fewer parasites were observed in two animals deprived of their lambs for 33 and 40 days, respectively (O'Sullivan and Donald, 1973). In immune responding animals, an increase in mast cells, eosinophil and GLs was recorded, whereas in lactating or pregnant ewes very few GLs were found (O'Sullivan and Donald, 1973). The same authors proposed that the proliferation and build-up of mast cells in the sub-epithelium was dependent on the production of differentiated effector lymphoid cells. The basis for this proposal was data presented by Dineen and Kelly (1972) where transfer of lymphoid cells from rats immune to *Nippostrongylus brasiliensis* to naive animals induced immunity in the recipients but the differentiation of induced cells to effector cells was inhibited in lactating rats.

Diet

Dietary protein intake can affect the development of resistance to nematode infection. Lambs (three months old) on a high protein intake, acquired immunity to *H. contortus* infective L3 trickle infection (200 L3, three times weekly) but those on a low protein diet did not develop resistance to the nematode and suffered a reduction in weight gain (Abbott, Parkin and Holmes, 1988). However, protein diet did not affect the ability of older lambs (>7 months) to become resistant to infection (Abbott and Holmes, 1990). Similar effects were observed with *T. colubriformis* infection (Kambara *et al.*, 1993). During parasitic infestation of the ovine GI tract protein intake is probably more crucial in young lambs as losses of plasma proteins into the gut lumen will increase demand (Poppi *et al*, 1986). Also, lymphocyte responsiveness to T cell mitogens was greatest in older lambs given higher protein levels in their diet (Poppi *et al.*, 1986).

1.7 WORM REJECTION

The immune expulsion of parasitic nematodes from the alimentary tract has been extensively reviewed (e.g. Miller, 1984; Rothwell, 1989) and two main processes of expulsion have been described. The first is rapid expulsion (RE) in which incoming larvae are prevented from establishing and are expelled shortly after infection. RE does not result in death of the nematode. The second is the expulsion of larvae and adult worms already established. The underlying mechanisms of rejection are unclear.

Rapid expulsion (RE)

The most studied examples of RE have been in the laboratory animal models of *Trichinella spiralis* (e.g. McCoy, 1940; Love, Ogilvie and McLaren, 1976; Bell and McGregor, 1979; Arasu *et al.*, 1994) and *N. brasiliensis* (Miller, Huntley and Wallace, 1981) infections. Following a secondary infection of *T. spiralis* in immune rats, the majority of the challenge larvae were expelled within 12 hours of oral administration (Bell and McGregor, 1979). Antibodies (IgG_1 and IgG_{2c}) from rat dams expressing RE are transferred to their off-spring via colostrum and within a few

hours of challenging suckling rat pups with *T. spiralis* L1 larvae, >90% of the worms were rapidly expelled (Appleton, Schain and McGregor, 1984; Appleton, Schain and McGregor, 1988). With *N. brasiliensis*, >85% of 4 day worms, which were introduced intraduodenally into rats immunised previously with L3 larvae, were expelled within the first 4 hours (Miller, Huntley and Wallace, 1981). In this latter study, RE was unaffected by the size of larval challenge given but diminished with time after the primary infection.

The effector mechanisms involved in RE are unclear, but secretion of mucus, has been proposed as one of the mediators involved (Lee and Ogilvie, 1981; Miller, Huntley and Wallace, 1981). *T. spiralis* larvae administered to immune rats became entrapped in mucus (Lee and Ogilvie, 1981). During a secondary challenge infection of *N. brasiliensis* in rats, the worms were prevented from migrating to their normal habitat between the villi by a superficial layer of mucus (Miller, Huntley and Wallace, 1981), though the worms were not actually immobilised by the mucus, as observed with *T. spiralis*.

Later work by Appleton, Schain and McGregor (1988), and Carlisle, McGregor and Appleton (1990, 1991 a and b) suggested that mucus itself may not be the primary mechanism in entrapment in RE of *T. spiralis*, but that this response may be mediated by antibodies. Binding of antibodies to the larvae may in some way impair their ability to penetrate the mucus layer to the epithelial tissue, or, the antibodies may actually bind to the mucus itself entrapping the worms as they enter the mucus (Appleton, Schain and McGregor, 1988; Carlisle, McGregor and Appleton, 1990, 1991 a and b).

RE has also been observed in the rejection of *T. colubriformis* from immune guinea-pigs (Rothwell and Love, 1974), furthermore, sheep developing resistance to repeated *T. colubrifomis* larval infections excreted increasing numbers of L3 larvae in their faeces (Chienjina and Sewell, 1974 a) suggesting RE. This was later confirmed by Emery *et al.*, (1992 a and b). Sheep immune to *T. colubriformis* were inoculated with *T. colubriformis* exsheathed L3 through the pylorus and expelled the challenge within 4 hr (Emery, McClure and Wagland, 1993).

Two mechanisms of RE were proposed for *T. colubrifomis* infections in sheep by McClure *et al.* (1992). Firstly, the rapid appearance of GLs at the site of infection suggested elimination in response to mast cell degranulation (McClure *et al.*, 1992), an idea supported by the increase in sheep mast cell proteinases monitored in the duodenal contents of immune sheep after challenge with *T. colubrifomis* L3 (results unpublished, cited in Emery, McClure and Wagland, 1993). The second is the leakage of *T. colubriformis* L3-specific IgG_1 and IgG_2 from the blood into the mucus (McClure *et al.*, 1992). The two processes could be linked if the increase in blood vessel permeability is caused by mast cell mediators (McClure *et al.*, 1992).

Expulsion of established larvae and adult

The *T. colubrifomis* worm burden that was not expelled by RE from immune sheep immediately after challenge was rejected 3 - 14 days p.i. (McClure *et al.*, 1992). Also, sheep immunised by adoptive transfer of adult *T. colubrifomis*, expelled most of their worm burden during the second week of infection after challenge with infective L3 (Emery *et al.*, 1992 b). This rejection was similar to expulsion of a primary infection at the adult developmental stages, suggesting that antigens that elicit rejection were stage-specific and were only present, or made in ample quantities when the parasites had developed for one week (McClure *et al.*, 1992).

McClure *et al.* (1992) suggested three possible mechanisms by which later rejection of established larvae and worms may be achieved. Increases in IgA and IgG_2 occur in the mucosa of immune animals 3 days post challenge infection and is probably accounted for by the production of *T. colubriformis*-specific antibodies locally (McClure *et al.*, 1992). During infection there are also changes in T cell migration, activation and effector function (McClure *et al.*, 1992). The role of antibodies and lymphocytes will be discussed further in the next section. The third mechanism is epithelial necrosis and sloughing which becomes evident after about 3-5 days, this is accompanied by an infiltration of lymphocytes (McClure *et al.*, 1992).

1.8 HUMORAL AND CELLULAR RESPONSES TO INFECTION

The transfer of serum or lymphoid cells from laboratory animals immune to parasitic infection to naive animals in an attempt to transfer resistance have been used to investigate the mechanisms of host immunity in several host-parasite systems. The transfer of both have been shown to induce immunity to *N. brasilienis*, *T. spiralis*, *Trichuris muris*, *T. colubriformis* and *Strongyloides ratti* (reviewed by Wakelin, 1978) with varying degrees of success. Immunisation by these methods invoked immune responses in naive animals but did not induce RE (Miller, 1984).

Attempts to passively immunise naive guinea-pigs with serum from animals immune to *T. colubriformis* infection did not prove successful (Wagland and Dineen, 1965). Conversely, the transfer of lymphocytes from guinea-pigs which were resistant to *T. colubriformis* to non-immune animals induced resistance to *T. colubriformis* challenge, leading Dineen and Wagland (1966) to hypothesise that immunity to *T. colubriformis* in guinea-pigs is a cellular phenomenon. Subsequent studies by Connan (1972) contradicted these results (Wagland and Dineen, 1965) as 3 out of 4 attempts to passively immunise guinea-pigs against *T. colubriformis* were positive. Connan (1972) suggested that in the previous experiment which examined passive transfer of immunity (Wagland and Dineen, 1965), guinea-pigs were immunised with light infections of the parasite, whereas, in the latter study (Connan, 1972) primary donors were given high worm burdens. However, Rothwell *et al.* (1980) and Adams, Merritt and Cripps (1980) were unable to repeat passive immunisation against *T. colubriformis* in guinea-pigs or sheep. Despite these results, the action of antibody and cellular responses in host immunity are invariably interconnected (Miller, 1984).

Humoral responses

Although immunisation by passive transfer has not been clearly demonstrated against *T. colubriformis* in the host, evidence does exist for stimulation of systemic and local antibody responses by *T. colubriformis* infection (Cripps and Rothwell, 1978; Adams, Merritt and Cripps, 1980).

Immune sheep which were challenged with *T. colubriformis* responded with increases in plasma cells that exhibited IgA and IgG_1 at the site of infection in the intestinal mucosa (Adams, Merritt and Cripps, 1980), with some antibodies having specificity for the parasite antigens. The population of IgA-secreting cells did not increase during challenge infection in areas of the intestine where worms were not residing. Also, IgA-secreting plasma cells increased as the percentage of IgA-containing lymphocytes rose, though a similar trend was not observed with the IgG_1 cells. However, IgG_1-secreting cells may be synthesised *in situ* rather than migrating through the lymphatic system (Adams, Merritt and Cripps, 1980). IgA cells in the lamina propria may be derived from IgA-containing lymphocytes from the intestinal lymph and blood, or, IgA-containing lymphocytes may accumulate in the lamina propria and differentiate to increase the numbers of IgA-secreting plasma cells (Adams, Merritt and Cripps, 1980). Wedrychowicz and Bezubuk (1988) also noted an increase in IgA and IgG during primary *T. colubriformis* infection in rabbits and found IgM to predominate following a third challenge infection. Local increases of IgA and IgG have been described during *H. contortus* (Smith, 1977; Duncan, Smith and Dargie, 1978; Gill *et al.*, 1993) and *O. circumcincta* infections in sheep (Smith *et al.*, 1984).

Secretory IgA molecules are capable of inducing eosinophil degranulation (Abu-Ghazaleh *et al.*, 1989). IgG antibodies have been shown to suppress *T. colubriformis* feeding *in vitro* (Bottjer, Klesius and Bone, 1985) and have also been implicated in mucus entrapment of parasite larvae (Carlisle, McGregor and Appleton, 1990,1991).

The anaphylactic antibody, IgE, also becomes elevated during parasitic infection but its role in host immunity is poorly understood. The IgE response is probably best defined in infection in man by *Schistosoma* spp.. Hagan *et al.* (1991) recorded high levels of anti-*Schistosoma* IgE in adults resistant to infection, whereas, susceptible children had low levels. Generally, levels of non-specific IgE increase in parallel with specific IgE, thus, the conventional allergic response may act in concert with the immune reaction against infection (reviewed by Hagan, 1993 and Pritchard, 1993 b). The allergic response is thought to have evolved as a consequence of

animals requiring IgE to combat helminth infections (Hagan, 1993). Pritchard (1993 b) suggested that parallel increases in non-specific IgE may compete with specific IgE and reduce the effect of the latter, favouring parasite survival. However, this may be required to protect the host from the detrimental effects of hypersensitivity reactions to parasite antigens (Pritchard, 1993 b). Eosinophils, mast cells, macrophages and platelets all have receptors with affinity for IgE and IgE is regulated by the opposing effects of the cytokines, IL-4 and IFN-γ (Hagan, 1993). Parasites possibly excrete or secrete components that are able to select T_H2 $CD4^+$ T cells, inducing proliferation of IgE producing B cells (Pritchard, 1993 b).

Cellular responses

Lymphocytes

Neonatal thymectomy and lymph drainage of guinea-pigs, thereby removing the thymic small lymphocyte population, led to loss of resistance to *T. colubriformis* in guinea-pigs which were previously immune to infection (Dineen and Adams, 1971). Removal of the thymus alone had no effect on the development of resistance, and long term lymph drainage had some effect but not as strong as when both treatments were applied, suggesting that thymus-dependent recirculating and long-lived cells were responsible for the initiation of worm expulsion (Dineen and Adams, 1971). Successful immunisation against GI nematodes conferred by the transfer of lymphocytes does not necessarily imply that the lymphocytes themselves are directly involved in the expulsion of the worms, as they may promote other effector mechanisms (Miller, 1984). Rothwell and Dineen (1972) noted that naive guinea-pigs, in receipt of mesenteric lymph node cells from *T. colubriformis* immune donors, responded to *T. colubriformis* challenge with a pronounced proliferation of bone marrow eosinophils and basophils and their accumulation and degranulation in the small intestine. This suggested an interaction of sensitised lymphoid cells with parasite antigens and associated changes in eosinophils and basophils. *et al.*, 1992).

The role of T_H cells and their cytokines in helminth infections has been widely studied (reviewed by Finkelman *et al.*, 1991; Finkelman and Urban, 1992), and is directly involved in eosinophilia, mastocytosis and basophilia, as well as in the appearance of B cells and goblet cells (Kassai, 1989). T_H1 cytokines, particularly, IFN-γ, appear to be involved in the control of parasites that spend some of their parasitic life intracellularly, such as *Leishmania major* infection in rats (Finkelman and Urban, 1992), whereas, T_H2 cells may be responsible for the control of extracelluar parasitic invasion, including *N. brasiliensis* (Finkelman *et al.*, 1991; Finkelman and Urban, 1992). The triggering mechanism for T_H2 inducing cytokines is not clearly defined. Recent research has suggested that proteolytic enzymes secreted by parasites may be involved (Finkelman and Urban, 1992). Injection of the cysteine proteinse, papain, into the foot-pad of BALB/C mice triggered an increased expression of messenger RNA (mRNA) for IL-4, IL-5 and IL-9 (T_H2 cytokines) but not IL-2 or IFN-γ (T_H1 cytokines) (Finkelman and Urban, 1992).

McClure *et al.* (1992) studied the various changes in lymphocytes during the course of *T. colubriformis* infection in susceptible and immune sheep. Following infection of non-immune lambs, slight increases in the local accumulation of T cells ($CD5^+$, $CD8^+$ and $CD4^+$) at the site of infection and a fall of plasma $CD8^+$ cells from 3 days p.i. was noted. This change was probably a result of a non-specific inflammatory response. Challenge of immune sheep with *T. colubriformis* resulted in an increase in T cells of all phenotypes in the lamina propria, and also elevated $CD8^+$ in the sub-epithelium, increases in $T19^+$ cells at the base of villi and 4-fold rise in $\gamma\delta^+$ cells throughout the lamina propria and epithelium. Blood changes included accumulation of $CD4^+$ and $CD8^+$ cells. All the elevated T cell responses peaked at 3 days p.i. (except for $CD5^+$, which peaked at day 5) and had decreased back to levels seen in non-challenged immune sheep by 14 days p.i. The accumulation of T cells observed in challenged immune sheep was accounted for by a specific antigenic response (McClure *et al.*, 1992).

Eosinophils, basophils, mast cells and globule leucocytes

Eosinophils, basophils and mast cells possess high affinity receptors for IgE and are important cellular components of the inflammatory response, releasing mediators which may induce permeability changes in the intestinal mucosa allowing 'leakage' of plasma macromolecules such as complement components or antibodies to reach the site of infection (Befus and Bienenstock, 1982; Huntley, 1992). Rothwell and Dineen (1972) studied the cellular responses in guinea-pigs following primary and secondary infections with *T. colubriformis*. Increases in bone marrow and circulating eosinophils and basophils as well as accumulation and degranulation of these cells were evident at the site of infection. Such changes occurred at a greater rate in animals receiving a secondary challenge with *T. colubriformis*, being maximal at 5-7 days p.i., compared to primary infected animals where cells peaked at 21-29 days p.i. These peaks correlated to expulsion of parasites in both groups. Further evidence that eosinophils play an essential role in immunity was provided by the observation that the administration of anti-eosinophil antiserum to guinea-pigs partially delayed the immune expulsion of *T. colubriformis* (Gleich *et al.*, 1979). However, Dineen and Windon (1980) found that intestinal eosinophil numbers could not be correlated with resistance to nematode infection. Dawkins, Windon and Eagleson (1989) demonstrated that circulating eosinophil numbers were generally higher in 'high-responder' lambs compared to 'low-responder' animals. Later research led to the conclusion that eosinophils, rather than being an indication of infection, were an indication of the ability of lambs to respond to *T. colubriformis* infection and vaccination (Buddle *et al.*, 1992). The precise role of eosinophils in nematode infections has not yet been defined, but in *Schistosoma mansoni* eosinophils have been demonstrated to damage the parasite *in vitro* (Glauert *et al.*, 1978).

As discussed earlier, Gregg *et al.* (1978) in their study of age-related unresponsiveness in sheep to *T. colubriformis* infection, provided evidence that resistance was not correlated to mast cells or eosinophil numbers, but to GL levels. This was confirmed by Stankiewicz *et al.* (1993) who showed that during infection in sheep, GLs were negatively correlated with worm burden and egg-production in infections with *H. contortus*, *T. colubrifromis*, *T. vitrinus* and *Cooperia curticei*.

Also, GLs were the only cells positively correlated to the inhibition of larval migration *in vitro* (Stankiewicz *et al.*, 1993). The exact origin of GLs that infiltrate the GI mucosa during nematode infection is still a matter of debate (reviewed by Huntley, 1992). Studies of GLs in ruminants and rats have suggested that mucosal mast cells migrate to the epithelium and discharge their contents at the tip of the villi, forming GLs that subsequently migrate to the submucosa and lamina propria (Murray, Miller and Jarrett, 1968; Miller and Jarrett, 1971). No migration of GLs from epithelium to the mucosa was observed. This morphological change from mast cells to GLs led them to be classified as transitional cells (Huntley, 1992). Conversely, Ruitenberg and Elgersma (1979, 1980) noticed the migration of GLs from epithelium to the lumen and suggested that GLs do not originate from mast cells, but move from the sub-epithelium to the epithelium.

Despite the observed mastocytosis during nematode infection of the GI, evidence for the functional involvement of mast cells in immunity is limited although the secretion of rat mast cell proteinase II during mast cell degranulation, as observed by increased serum and intestinal levels of the enzyme, coincides with the immune expulsion of *T. spiralis* and *N. brasilensis* during primary infections in the rat (Woodbury *et al.*, 1984). Sheep mast cell proteinase (SMCP) has also been observed in the duodenal contents of immune sheep challenged with *T. colubriformis* (unpublished results, cited in Emery, McClure and Wagland, 1993), and in the abomasal tissue and gastric lymph of immune sheep infected with *H. contortus* (Huntley *et al.*, 1987; unpublished results, cited in Emery, McClure and Wagland, 1993). *In vitro* incubation of mucosal mast cells from immune sheep with extracts from *T. colubriformis*, *H. contortus*, *T. vitrinus* or *O. circumcincta* and recombinant antigens from *T. colubriformis* and *H. contortus* stimulates the secretion of SMCP (unpublished results, cited in Emery, McClure and Wagland, 1993). The role of mast cell proteinases was reviewed by Huntley (1992) and are thought to be associated with change in the permeability of the intestinal mucosa, mucus secretion and shedding of the epithelium, all of which would enhance migration of plasma effector components to the intestinal lumen.

Inflammatory mediators

Cells which have become sensitised by exposure to nematode antigens release a variety of mediators which have been implicated in expulsion of parasites (Douch *et al.*, 1984). Inhibition of histamine and 5-hydroxytryptamine (5-HT) by drugs impaired the capacity of immune guinea-pigs to reject a *T. colubriformis* challenge infection (Rothwell, Dineen and Love, 1971) and administration of histamine/histidine and 5-HT to non-immune guinea-pigs resulted in the development of immune responses to *T. colubriformis* challenge (Rothwell, Dineen and Love, 1971; Rothwell, Prichard and Love 1974) with significant worm expulsion at days 5-9 p.i.. Amine dosage during only the first 7 days of infection (L3/early L4) or days 8-12 p.i. (late L4 stage) did not affect the outcome of infection suggesting that the amines must be present throughout the entire L4 stage to cause expulsion (Rothwell, Prichard and Love 1974). A biphasic mechanism of resistance was suggested by Rothwell, Dineen and Love (1971). The first step is immunologically specific and involves immune lymphocyte-parasite antigen interaction, leading to accumulation and degranulation of basophils and eosinophils with the release of mediators such as amines. The second, and immunologically non-specific, step is the final effector mechanism and involves rejection of the parasite mediated by the activity of amines.

Douch *et al.* (1984) compared the histamine levels in sheep which were defined as high and low responders to *T. colubriformis* infection. No significant differences in histamine levels were detected in the fluid contents of the small intestine or abomasa when high and low responders were compared and these levels could not be correlated to parasite elimination. Histamine levels were greatest in the gut when the highest numbers of worms was present.

During *T. colubriformis* infection in sheep, Jones and Emery (1991) recorded the intestinal mucosal levels of histamine, leukotriene C_4, 6-keto-prostaglandin $F_{1\alpha}$ (from prostacyclin) and thromboxane B_2. Following the challenge of immune sheep with *T. colubriformis* infection, the levels of these mediators in the intestinal mucosa increased in the first six days after challenge, with leukotriene C_4 being released in the largest quantities. Histamine is a granular mediator and the other mediators are mast

cell membrane-associated mediators metabolised from arachidonic acid (Jones and Emery, 1991). Leukotriene C_4 is also a major mediator released by eosinophils.

1.9 CURRENT CONTROL PROCEDURES

Anthelmintic drugs are at present the primary means for control of PGE. Three types are currently available, though their modes of action are not clearly understood: benzimidazoles (BZ) bind to parasite tubulin, limiting polymerisation of α and β subunits and affect metabolism (Lacey and Prichard, 1986); the levamisole/morantel family are anticholinergic drugs disrupting sodium transport (Coles, East and Jenkins, 1975); and the most recent, the ivermectins, bind to specific receptors increasing the permeability of glutamate-gated chloride channels (Prichard, 1990; Cully *et al.*, 1994). The latter two cause paralysis of the parasite (Prichard, 1990). All have broad spectrum activity against most GI nematodes, as well as some other tissue worms and do not have any adverse effects on the host.

Farmers are advised to dose animals regularly with anthelmintics even though no clinical signs of nematode infection are evident, thus using the drugs in a preventative manner. However, even light infections cause substantial losses in production and administration of anthelmintics only removes the worm burden at time of treatment. Also, the duration of anthelmintic efficacy is short-lived and treated animals are susceptible to re-infection and, hence, the adverse effects of chronic parasitism (Prichard, 1990).

To improve the effectiveness of anthelmintics, the drugs should be used in conjunction with grazing management schemes (Waller, 1987), enabling residual pasture contamination to diminish prior to reintroduction of stock onto the pasture with limited risk of re-infection. This is achieved by letting the pasture rest, rotational grazing or alternate grazing with either different species or younger animals with older ones (Morley and Donald, 1980). However, grazing management is not always an economically valid option and is often not feasible, especially on hill farms, due to the limited availability of suitable grazing.

1.10 DISADVANTAGES OF ANTHELMINTICS

A major threat to the future control of PGE is the development of anthelmintic resistance in nematode populations. Resistance has been recorded in many of the principle nematode species, to all the currently available anthelmintics (Prichard, 1990) and is the cause of world-wide concern. Nematode resistance to BZ is the most common in the UK, while very little resistance to the other two groups of anthelmintics has been found as of yet (Jackson, 1993). A survey of sheep farms in England (Coles, Hong and Hunt, 1991) and Scotland (Mitchell, Jackson and Coop, 1991) recorded prevalence rates at 51% and 24%, respectively. Resistance to ivermectin has also been shown in a strain of *Ostertagia* spp. isolated from hill goats in Scotland, these nematodes were resistant to BZ as well (Jackson *et al.*, 1992). The occurrence of resistance in nematodes is thought to be a pre-adaptive phenomenon, the genes for resistance being already present in small numbers within the normal nematode population gene pool and the use of anthelmintics selecting nematodes carrying these resistance genes. This is in contrast with the occurrence of resistance through random mutations (reviewed by Jackson, 1993). Resistance to BZ occurs due to changes in the parasite β-tubulin isotype pattern, resulting in the loss of the BZ high affinity receptor binding sites (Conder and Campbell, 1995; Roos, Kwa and Grant, 1995). There are at least two mechanisms involved: initially, there is a loss in β-tubulin isotype 1 alleles (conferring low to moderate levels of resistance), and this is followed by the loss of different isotype 2 alleles (conferring high levels of resistance) (reviewed by Conder and Campbell, 1995; Roos, Kwa and Grant, 1995). The molecular mechanisms of parasite resistance to ivermectin and levamisole/morantal anthelmintics are not so well defined, however, it is unlikely that a single step is involved (reviewed by Conder and Campbell, 1995).

New drugs are very expensive to develop so manufacturers are trying to maintain usage of drugs already on the market by creating derivatives. However, in addition to the problem of anthelmintic resistance there is also growing concern about the presence of drug residues in meat and their effect on the environment. Residues

excreted in the faeces have the potential to interfere with soil fauna and arthropods feeding on the faeces (Strong, 1993; Waller and Larsen, 1993).

The control of PGE in the immediate future will still rely mainly on anthelmintic drugs but there is a need to develop alternative control procedures.

1.11 ALTERNATIVE CONTROL PROCEDURES

Biological control

Nematophagus fungi are microfungi which capture nematodes either as a source of food or supplementary to a saprophytic existence. The potential use of these fungi as a method for controlling the free-living stages of animal parasitic nematodes was reviewed by Waller and Larsen (1993). Gronvold *et al.* (1987, 1993) demonstrated that isolates of the fungi, *Arthrobotrys* spp. trapped the free-living larvae of several parasitic nematodes and some soil-living nematodes. These fungi are also capable of surviving passage through the ruminant gut and are excreted in the faeces together with nematode eggs (Waller, 1993 a). For example, some isolates of *Arthrobotrys* and *Duddingstonia* survived passage through calves and significantly reduced the numbers of developing nematodes in the faeces (Larsen *et al.*, 1992; Gronvold *et al.*, 1993). Such biological control does not completely eliminate the target parasite but reduces pasture contamination to practically acceptable levels. Small numbers of nematodes would still allow the host to develop natural immunity through trickle infection. The main disadvantage of this type of biological control is that it is difficult to predict how the fungi will effect the established ecosystem, for example, they may also destroy beneficial soil nematodes.

Breeding for genetic resistance to nematodes

Resistance to nematodes varies between and within breeds of sheep. The black belly sheep of the Caribbean has evolved to become resistant to helminths due to exposure to high larval numbers over many years. However, they are not suitable for commercial use as they are small in stature with poor wool quality, as is often the case with resistant breeds (Waller, 1993 b). Variable susceptibility to nematodoses

within breeds was demonstrated by Dineen, Gregg and Lascelles (1978). After vaccination with irradiated infective L3 larvae of *T. colubriformis*, Merino lambs could be segregated into animals which had worm burdens similar to immune mature sheep ('high-responders') and those that had worm burdens comparable with unvaccinated animals ('low-responders'). Also, worm burdens acquired by permanently grazing sheep indicated some sheep were able to resist infection earlier than others (Douch *et al.*, 1984).

Selective breeding would allow an overall reduction in susceptibility of a flock. Factors to take into consideration for breeding programmes include cost effectiveness, the heritabilty levels and their relationship to economically important traits such as wool quality and growth rate. Non-specific resistance against all parasitic worms would be advantageous but should not affect non-pathogenic organisms, and the ability of parasites to develop resistance should remain within reasonable levels (reviewed by Albers and Gray, 1986 a and b; Windon, 1990, 1991). The identification of predictive markers for resistance is a prerequisite for a breeding programme and haemoglobin type appears to be associated with this trait (Altaif and Dargie, 1978 a and b; Windon, 1990, 1991). Outteridge, Windon and Dineen (1985) showed that a particular ovine lymphocyte antigen was found to be present in high numbers in 'high-responder' sheep and relatively few numbers in 'low-responder' animals. The lymphocyte antigen is thought to be part of the major histocompatability complex (MHC) which fundamentally influences the immune response to invading pathogens, including nematode parasites (Outteridge, Windon and Dineen, 1985). Improvement of phenotypic traits by genotypic selection will probably not completely negate the need for the present control procedures but should prolong the effectiveness of current anthelmintics due to the reduction in their use (Windon, 1990, 1991).

Immunological control

Control of parasitic nematodes by immunologically-based procedures should be feasible as sheep continually exposed to infection do develop immunity to subsequent infection (Chienjina and Sewell, 1974 a and b; Waller and Thomas, 1981;

Jackson, Angus and Coop, 1983), as discussed in section 1.6. The remaining part of this literature review, will deal solely with the prospects of vaccine control for parasitic infections.

1.12 VACCINATION

Live-attenuated vaccines

One early and successful approach to immunological control of helminth infections was the use of gamma-irradiated infective third stage larval vaccines that mimic immunity induced by natural infections. They produce self-limiting infections of about four weeks duration, effective immunity being stimulated primarily by antigens produced by L3, L4 and immature adults (Lloyd, 1981; Emery *et al.*, 1992 a & b). In 1958, Jarrett *et al.*, showed that the administration of two doses of *Dictyocaulus viviparus* L3 larvae, attenuated by gamma-irradiation, induced up to 98% protection against challenge infection with the parasite. This led to the development of the only commercially successful helminth vaccine, 'Dictol', which protects against the bovine lungworm, *D. viviparus* (Jarrett *et al.*, 1958). Following this, an irradiated larval vaccine was developed in India for the control of the ovine lungworm *Dictyocaulus filaria* (Dhar and Sharma, 1981; Sharma, Bhat and Dhar, 1988). A similar vaccine, based on irradiated L3 larvae, was introduced for the control of the canine intestinal nematode, *Ancylostom caninum* (Miller, 1971), but was withdrawn after only two years due to respiratory side effects, cost and short-shelf life. Also, upon re-infection although animals were protected from clinical infection, hookworm eggs were still observed in the faeces (Miller, 1978).

Attempts to vaccinate lambs against *T. colubriformis* using irradiated L3 larvae were successful for lambs older than six months of age (90% reduction in worm burden) but not for 3 month old lambs (37 % reduction in worm burden) (Gregg and Dineen, 1978; Gregg *et al.*, 1978). Serum antibody levels, peripheral cellular responses, mast cell, neutrophil and eosinophil responses were monitored in lambs of both age groups but no differences were observed (Gregg and Dineen, 1978; Gregg *et al.*, 1978). The only observed difference between the response in the two

groups was that the level of GLs in duodenal sections were significantly elevated in the older immune lambs. Similar results were seen with *H. contortus* (Urquhart *et al.*, 1966). For *Dictyocaulus* two doses of 10^2 - 10^3 irradiated L3 are required but with *T. colubriformis* and *H. contortus* individual doses of 2 x 10^4 irradiated larvae were necessary. The large quantity of irradiated L3 required in the latter cases and the age related unresponsiveness to the vaccine means that this type of vaccination is not feasible for *T. colubriformis* and *H. contortus* infections (Emery, McClure and Wagland, 1993).

Antigen vaccines

Immunity against parasitic infections is a result of recognition of parasite antigens by the host (Emery and Wagland, 1991). A previous problem with the development of defined antigen vaccines was that they could not be produced in sufficient quantities for practical purposes (Lightowlers, 1994). *In vitro* culture procedures for helminth parasites were costly due to the complexity of the culture media required, while *in vitro* antigen yields tended to be lower than those obtained from parasites harvested from the natural host (Emery, McClure and Wagland, 1993). In addition, to produce a single vaccine dose of antigen from parasites *ex vivo* usually requires the sacrifice of three donor sheep (Emery, McClure and Wagland, 1993). With the development of molecular biological techniques, the large-scale production of proteins for vaccination is now possible (Maizels and Selkirk, 1988) and research into irradiated vaccines has now been superseded by the search to develop vaccines containing purified antigens.

Identification of host-protective antigens is very difficult. There are three different sources of potential target antigens from the parasite: somatic or tissue proteins, cuticular/surface proteins and proteins that are excreted or secreted. At each stage of the nematodes parasitic life cycle (L3, L4, immature adult and mature adult) different antigens may be presented stimulating quite distinct host immune responses (Miller, 1984).

Protective antigens may be of two types, firstly, those that play an active role in the development of immunity following natural infection. Such conventional

antigens include the glutathione-S-transferase (GST) from *Schistosoma* spp. (Smith *et al.*, 1986). However, mutations could occur in the genes encoding these conventional antigens and host immunity may exert a selective pressure which would favour the rapid selection of parasites carrying these mutations thus rendering the vaccine ineffective in a manner similar to the onset of anthelmintic resistance (Emery and Wagland, 1991). Moreover, host recognition of antigens which stimulate natural immunity may be restricted by elements of the MHC (Kennedy, 1991). The second category includes antigens that are not normally host-protective but induce host protective responses if presented correctly by vaccination; covert antigens. The immune response obtained by vaccination of 97 kDa paramyosin to *Schistosoma* (Lanar *et al.*, 1986) and the 41 kDa tropomyosin from *T. colubriformis* (O' Donnell *et al.*, 1989 a) exemplify these covert antigens. Repeated vaccination is required for maintenance of immunity because host recognition of covert antigens is not boosted by natural infection (as would occur with the conventional antigens) (Emery and Wagland, 1991).

1.13 EXCRETORY-SECRETORY COMPONENTS

Vaccination with excretory/secretory components (ES) was shown to stimulate host protective immune responses against several nematodes including *T. colubriformis* (Rothwell and Love, 1974; O'Donnell, 1989 b), *T. spiralis* (Vernes, 1976), *Ascaris suum* (Stromberg and Soulsby, 1977), *N. brasiliensis* (Day *et al.*, 1979) and *D. viviparus* (McKeand *et al.*, 1995).

ES is defined as the components that are actively shed from the cuticle, as well as materials released from specialised excretory-secretory organs (Lightowlers and Rickard, 1988). Active shedding of surface proteins by *T. spiralis* was demonstrated by Phillip, Parkhouse and Ogilvie (1980). Also, Maizels, Meghji and Ogilvie (1983) showed that the surface protein composition of *N. brasiliensis* L3 completely changed in the course of the 18 hr normally taken from first penetrating the host to reaching the lungs, without an observable moult. Shedding of the cuticle surface molecules may remove antibodies, complement and host immune cells from the

parasite, forming part of an immune evasion strategy. However, this may also make previously concealed surface components accessible to antigen presenting cells, thus stimulating an immune response (Lightowlers and Rickard, 1988).

The cuticle of L3 and adult *T. spiralis* and *N. brasilensis* have been shown to activate complement via the alternative pathway and rats infected with these parasites produce specific antibodies to all the stages in the parasite's life cycle (Mackenzie *et al.*, 1980). Also, *in vitro*, complement killed infective *N. brasilensis*, and an eosinophil-enriched cell population and antibodies were detrimental to infective and adult *T. spiralis* (Mackenzie *et al.*, 1980). ES molecules capable of binding complement, and antibody directed against parasite surface antigens have been demonstrated in *Toxocara canis* (Badley *et al.*, 1987).

The secretion of enzymes by parasitic nematodes *in vitro* is well documented and have been implicated in evasion of many host immune responses. AChE is secreted by numerous nematodes, including the L4 and adult stages of *T. colubriformis* (Ogilvie *et al*, 1973; Rothwell, Ogilvie and Love, 1973) and adult *T. vitrinus* (Jones and Knox, 1990). AChE has been assigned several roles in maintenance within the host including acting as a 'biochemical holdfast', inhibiting local gut peristalsis (Lee, 1970), reduction of mucus secretion (Philipp, 1983) and in the control of cellular immune responses (reviewed by Philipp, 1983; Rhoads, 1984; Pritchard, 1993 a).

Numerous proteinases have been defined in ES. Anticoagulant proteinases have been identified in *A. caninum* (Hotez and Cerami, 1983 ; Hotez *et al.*, 1985) and *O. circumcincta* (Young, Knox and McKeand, 1995), and the blood-feeding nematode, *Strongylus vulgaris* (Caffery and Ryan, 1994), secretes haemoglobinases. Cleavage of IgG by ES proteinases from *S. mansoni* schistosomulae (Auriault *et al.*, 1981) and the trematode adult *Fasciola hepatica* (Chapman and Mitchell, 1982) provide more direct evidence of ES proteinase involvement in evasion of host effector mechanisms. Also, peptides produced due to the IgG cleavage by *S. mansoni* schistosomulae can play a role in regulating IgE synthesis (Verwaede *et al.*, 1988). The implications of AChE and proteinase secretion by nematodes will be discussed more fully in chapter three.

In addition to proteinases, proteinase inhibitors have been detected in the ES of parasites. A cysteine proteinase inhibitor has been detected in the ES from adult *Onchocerca volvulus* (Lustigman *et al.*, 1992). Furthermore, *Echinococcus granulosus* (dog tapeworm) cyst stages secrete a serine proteinase inhibitor capable of preventing neutrophil chemotaxis and inhibition of elastase activity (Shepherd, Aitken and McManus, 1991). Proteinase inhibitors have also been detected in parasite homogenate material. For example, *Taenia taeniaeformis* (Leid *et al.*, 1986) has been reported to contain a proteinase inhibitor capable of inhibiting endogenous IL2-1-induced proliferation of murine thymocytes, and extracts of *A. suum* and *A. lumbricoides* inhibit the proteolytic activity of human pancreatic extracts (Hawley and Peansky, 1992). Host proteinases are involved in several plasma activation cascades such as the role of the serine proteinase, thrombin, in the clotting process, and many leucocytes, for example mast cells, release proteinases which have roles in activation-secretion processes (Leid, Suquet and Tanigoshi, 1987). Clearly, it is to the parasite's advantage to evolve mechanisms that inhibit these processes.

The release of enzymes that mop up free oxygen radicals released by phagocytic cells during the inflammatory response, with the potential to reduce oxidant damage to the invading parasite has been reviewed by Callahan, Crouch and James (1988) and James (1994). Knox and Jones (1992) noted that superoxide dismutase (SOD) activities and isoenzyme polymorphism were higher in 'adapted' *N. brasiliensis*, retrieved from immune rats, compared to normal worms from naive rats.

Immunosuppressant components in ES have been suggested by observations on the *in vitro* immunological properties of ES from several parasites including *S. mansoni* (Dessaint *et al.*, 1977), *T. spiralis* (Faubert, 1976), *Onchocerca gibsoni* (Foo *et al.*, 1983), *Oesophagostomun radiatum* (Gasbarre, Romanowski and Douvres, 1985) and *Heligmosomoides polygyrus* (Pritchard *et al.*, 1994). The antigen-specific proliferation of lymphocytes that were primed with either keyhole limpet haemocyanin or ovalbumin, as well as proliferation induced by T cell mitogen concanavalin were inhibited by the ES components of L3 and L4 *O. radiatum* (Gasbarre, Romanowski and Douvres, 1985). Immunosuppression of host protective

responses may reduce the ability of the host to build up resistance to subsequent infections (Gasbarre, Romanowski and Douvres, 1985).

Parasites, including the ovine GI nematode, *H. contortus* (Kapur and Sood, 1991), also release a variety of lipids during culture. The role of lipids in parasite maintenance is unkown, but secretory fatty lipids from *Schistosoma japonicum* (Asahi *et al.*, 1984) induce haemolysis, a function which could be of relevance to the blood sucking *H. contortus* (Kapur and Sood, 1991).

Given the proposed roles of parasite ES components in parasite survival, nematode ES may be a major source of host protective antigens, and therefore, candidate target antigens for vaccine development.

1.14 PROTECTIVE ANTIGENS ISOLATED FROM *TRICHOSTRONGYLUS COLUBRIFORMIS*

The identification of potential host-protective antigens of *Trichostrongylus* spp. has, to date, centred on *T. colubriformis*, most of the associated research being carried out by groups in Australia. The work of Emery *et al.* (1992 a and b) and McClure *et al.* (1992) demonstrated that both RE and late rejection resulted in reduced egg production from immune sheep compared to non-immune controls following challenge with *T. colubriformis*. This suggested that antigens from larval or adult stages of the parasite could have utility for subunit vaccine preparations, reducing the clinical effect of parasitosis as well as pasture contamination.

Early experiments (Rothwell and Love, 1974; Rothwell and Merritt, 1975) demonstrated that injection of somatic and ES proteins from L4 and adult *T. colubriformis*, but not the L3, could stimulate resistance in guinea-pigs (the laboratory model for this infection), with mature animals responding better than younger ones. Studies on the localisation of protective antigens of *T. colubriformis* by Rothwell and Sangster (1991) did not produce particularly clear results, but suggested that *T. colubriformis* protective antigens were widely distributed in the body of the nematode, and not concentrated in the intestine or glandular structures. Vaccination of guinea-pigs (O'Donnell *et al.*, 1985), with homogenates of L4 *T.*

colubriformis caused accelerated expulsion of worms in guinea-pigs following homologous challenge in agreement with the results of Rothwell and Love (1974), and subfractions of this homogenate (67 - 94 kDa) isolated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were also shown to accelerate parasite expulsion (O'Donnell *et al.*, 1985). The protein content of these fractions were still very complex and subsequent work aimed to identify the specific protective antigens.

Phosphate-buffered saline/sodium-deoxycholate extracts of third stage *T. colubriformis* larvae were found to contain four major proteins (O'Donnell, 1989 a). Three of the components, the 200 kDa (myosin-like), 93 kDa (albumin-like) and 43 kDa (actin-like) proteins, were not host protective. The fourth, a 41 kDa protein, induced 43-45% protection in guinea-pigs and was present in L3, L4 and adult stages of the parasite. Peptide sequencing of the 41 kDa protein revealed its homology to tropomyosin. Frenkle *et al.* (1989) cloned the gene encoding this protein and showed that it had 58% amino acid identity to tropomyosin from *Drosophila* and rabbit and overall. Tropomyosin did not stimulate an antibody response in the systemic circulation of naturally infected sheep or guinea-pigs and lymph from sheep infected with *T. colubriformis* did not contain antibodies to the 41 kDa protein. A muscle protein, paramyosin, has also been found to be protective against *Schistosoma* (Lanar *et al*, 1986). A 27 kDa sub-unit of the protein was expressed as a fusion protein of β-galactosidase in *Escherichia coli* (*E. coli*) and vaccination of guinea-pigs with the fusion protein led to accelerated worm expulsion following challenge with *T. colubriformis* (Cobon *et al.*, 1989). A gene encoding a related protein from *H. contortus* has also been identified and the expressed gene product was found to significantly reduce worm burdens in immunised sheep following challenge with *H. contortus* (Cobon, *et al.*, 1989). However, the level of protection achieved by vaccination with recombinant tropomyosin was not as strong as that attained during natural infection (Rothwell and Love, 1974), suggesting the requirement to identify more effective antigens or combinations of antigens and to identify the most appropriate mode of administration. Further research has analysed potential host-protective antigens from the ES of different stages of *T. colubriformis*.

The expulsion of worms from immune guinea-pigs upon challenge with *T. colubriformis* commenced within the first two days, suggesting components of the L3 stage activate the host immune response (Rothwell and Griffiths, 1977). Chiejina and Sewell (1974 b) also noted the early rejection of *T. colubriformis* in L3 from highly resistant sheep. These results prompted O' Donnell *et al.* (1989 b) to assess the host protective properties of ES components from exsheathed third-stage *T. colubriformis* larvae. The products of XL3 of *T. colubriformis* confer some protection (46-50%) against homologous challenge in guinea-pigs (O'Donnell *et al.*, 1989 b). The dominant protein isolated from the L3 ES was found to be a 94 kDa glycoprotein which was antigenic being strongly recognised by serum antibodies from naturally infected sheep and guinea-pigs (O'Donnell *et al.*, 1989 b). The protein was found to be associated with the cells of the alimentary canal of XL3 but not present in adult or L4 worms. An antigenically related 90 kDa protein is present in XL3 *H. contortus* (O'Donnell *et al.* 1989 b).

A major secretory protein, a 30 kDa lentil-lectin binding glycoprotein, from the L4 and adult stages of *T. colubriformis* ES, was found to stimulate a 59% level of protection in immunised guinea-pigs (Savin *et al.*, 1990). SDS-PAGE and Western blot results showed the apparent size of the protein to be actually in the region of 15-17 kDa. Northern blot analysis showed that the protein was predominately expressed in the L4 stage of the parasite, with lower levels detected with adult (Savin *et al.*, 1990). Full length cDNAs encoding the protein, isolated from a L4 *T. colubriformis* cDNA λgt11 library, had variable sequences suggesting the protein was encoded by a multigene family which may be expressed in response to different environmental stimuli, or, may reflect allelic variation within a nematode population. The protein shared 28% amino acid identity and 64% amino acid conservation with a putative precursor of porcine intestinal peptide, valosin. Valosin has been shown to increase secretions of gastric acid and pepsin from the stomach in dogs and significantly delay duodenal motility (Savin *et al.*, 1990). Therefore, this protein may be responsible, at least in part, for the inhibition of peristalsis in the gut during *Trichostrongylus* infestation (Savin *et al.*, 1990; Foster, Dean and Lee, 1993).

From the non-bound fraction of the lentil-lectin affinity column, Dopheide *et al.* (1991) isolated a 11 kDa non-glycosylated from the ES of L4 and adult *T. colubriformis*. The protein was used to immunise guinea-pigs. Sixteen out of eighteen animals were protected by greater than 50% following challenge with *T. colubriformis*. This was in comparison with non-immunised control animals, none of which were 100% protected and only three out of twenty-five showed more than 50% resistance to *T. colubriformis* infection. No antibodies to the protein were found in serum from naturally infected sheep or guinea-pigs. However, the possibility that the protein stimulates a cellular immune response in the course of a natural infection cannot be excluded. Northern blot analysis confirmed that the protein was expressed by both the L4 and adult stages of *T. colubriformis*. Sequence analysis found that the 11 kDa protein had homology to human gamma interferon-induced protein.

Frenkle *et al.* (1992) isolated a non-glycosylated 18 kDa protein from a non-binding lentil-lectin column fraction of ES from young adult *T. colubriformis* which conferred 60-84% protection in guinea-pigs challenged with *T. colubriformis*. Northern blot analysis revealed that the 18 kDa protein was expressed in L3, L4 and young adult stages of *T. colubriformis*, with the strongest expression in young adults. A full-length cDNA encoding the 18 kDa protein was isolated from a *T. colubriformis* immature adult cDNA λgt11 library and sequence analysis showed the protein was a globin-like molecule, with 20% amino acid identity with human α-globin and 21% identity with globin from the insect, *Chironomus thummi thummi*. The authors (Frenkle *et al.*, 1992) suggested that the molecule may play an essential role in oxygen transport.

From the lentil-lectin column bound fraction, referred to earlier (Savin *et al.*, 1990), Verkuylen *et al.* (1993) isolated a proline rich 37 kDa ES protein from adult *T. colubriformis* which was one of the prominent antigens recognised in immunoblots by lymph from naturally infected sheep. Sequence analysis of the full length gene encoding this protein did not match any sequences recorded in the computer databases.

The Northern blot analysis of mRNA from different stages in the life-cycle of *T. colubriformis* demonstrated that expression of the 94 kDa (O'Donnell, 1989 b), 30

kDa (Savin *et al.*, 1990) and 11 kDa (Dopheide *et al.*, 1991) ES proteins are under developmental control. This supports the suggestion that different nematode antigens are presented to the immune system by each parasitic stage within the host. Also, the work on *T. colubriformis* antigens demonstrates the potential of both conventional (e.g. Verkuylen *et al.*, 1993) and covert (e.g. Dophiede *et al.*, 1991) antigens in inducing host immune responses. Emery, McClure and Wagland (1993) cite unpublished results stating that recombinant conventional antigens of the 11, 17, 30 and 37 kDa ES proteins stimulated 30 - 60% protection in lambs against *T. colubriformis* infection and presumably these proteins correlate to those described above. However, details of these experiments have yet to be published.

1.15 RECOMBINANT ANTIGEN VACCINES

The practical utility of recombinant parasite antigens as antiparasite vaccines in the near future is supported by the granting of provisional registrations for recombinant antigen vaccines for the control of the cestode, *Taenis ovis* in August 1990, and the tick, *Boophilus microplus* in July 1992 (Lightowlers, 1994). The former is an example of vaccination with a conventional antigen that is recognised by the naturally infected host. The latter demonstrates the potential of utilising covert antigens to stimulate host protection.

Johnson *et al.* (1989) gave the first description of a highly effective recombinant vaccine against a parasite, *T. ovis* in sheep. Sheep acquire strong immunity to *T. ovis* and sera from immune sheep strongly recognise antigens derived from *T. ovis* oncospheres and their ES products. These antigens include a doublet at 47/52 kDa on Western blots. The oncosphere antigens were separated by SDS-PAGE and a gel slice including the 42/52 kDa doublet was removed and used to vaccinate lambs. These animals showed 98% protection to subsequent challenge with *T. ovis*. Rabbit antiserum was raised to the ES components of oncospheres and antibodies to the 47/52 kDa were selected by elution from Western blots. Immunoscreening of *T. ovis* oncosphere cDNA expression library identified two clones termed 45S (strong signal recognition) and 45W (weak signal recognition).

These cDNAs were expressed as β-galactosidase fusion proteins but vaccination with these fusions failed to stimulate protection in lambs, even though an IgG antibody response to the native 47/52 kDa doublet was stimulated. The cDNAs were subcloned into an alternative plasmid vector in which the antigens were expressed as fusion proteins with the enzyme GST of *S. japonicum*. The GST-45W fusion protein, using saponin adjuvant, stimulated 94% protection in lambs, however, the GST-45S fusion did not elicit any protective response. Sequence analysis of the two clones revealed that 45W was 500 base pairs longer than the 45S, which, therefore, lacked some of the protein encoding region. Treatment of the GST-45W fusion protein with SDS or dithiolthreitol markedly reduced the ability of the protein to protect indicating that protein conformation was an important part of antigen recognition. Although the levels of protection achieved were not 100%, the use of the antigen as a vaccine should enable substantial control of cysticercosis with obvious benefit to the sheep industry.

Boophilus microplus is a tropical cattle tick which is of economic importance in several parts of the world. Naturally acquired immunity in cattle to *B. microplus* is only partially effective, therefore, vaccination with conventional antigens would be unlikely to provide a means of protection. Research has focused on the identification of covert antigens. Johnston, Kemp and Pearson (1986) showed that crude extracts of *B. microplus*, which were removed from the host 24 hr before engorgement, reduced establishment by the tick on cattle immunised with the extracts. Subfractionation of these crude extracts by centrifugation established that material sedimented at 105,000 g, which included membrane components, gave the greatest protection (Willadsen, McKenna and Riding, 1988). This fraction was subfractionated by extensive separation procedures and vaccination trials identified a minor cell membrane 89 kDa glycoprotein as the effective antigen (Willadsen *et al.*, 1989). This protein is expressed on the surface of tick gut digestive cells. The protein was purified further and subsequent protection experiments resulted in a 65% reduction of ticks surviving to the engorgement stage while the weight of the remaining engorged ticks was reduced by 33% and egg production by females was reduced by 54%. Using oligonucleotides, an almost full length cDNA encoding the

89 kDa was isolated (Willadsen *et al.*, 1989; Rand *et al.*, 1989). Sequence analysis showed that the protein shared homology to epidermal growth factor in many regions. The cDNA was expressed as a β-galactosidase protein in *E. coli* as inclusion bodies and the protective potential of the recombinant antigen assessed. Though a high proportion of *B. microplus* were damaged only 24% of the tick population was killed by the vaccination. However, there was a significant reduction in the weights of the surviving ticks and, overall, there was a 77% decrease in the reproduction performance of the females.

The most promising candidate vaccine antigen to a GI nematode to date has been isolated from the blood-sucking parasite, *H. contortus*. A major component of the intestinal microvillar membrane in *H. contortus* is a 110,000 kDa integral glycoprotein, designated H11 (Smith and Munn, 1990). Sheep, immunised with a 'concanavalin A binding protein' fraction, 85% of which consisted of H11, stimulated significant protection against *H. contortus* in both mature sheep and 2 month old lambs, which would not normally express a protective immune response (Travernor *et al.*, 1992 a and b). Isolation and characterisation of the full length cDNA encoding the protein, revealed that H11 showed 32% amino acid identity and 52% amino acid conservation to mammalian microsomal aminopeptidase (personal communication, Dr. D.P. Knox, Moredun Research Institute, Edinburgh, UK). Enzyme analysis of purified native H11 showed that the protein was, indeed, an aminopeptidase (personal communication, Dr. D.P. Knox, Moredun Research Institute, Edinburgh, UK). The cDNA encoding H11 has been expressed in baculovirus and the protective ability of the resulting recombinant antigen is being assessed in vaccine trials (personal communication, Dr. D.P. Knox, Moredun Research Institute, Edinburgh, UK).

Following the identification of a suitable protective recombinant antigen vaccine against parasite infection, for a vaccine to be developed commercially, it must be easy to administer, provide long-lasting protection and be cost effective to be able to compete with current control procedures (Emery, McClure and Wagland, 1993; Lightowlers, 1994). Administration of antigen vaccines against GI nematodes directly to the GI, may be desirable in some situations where the only identifiable protective immune responses are expressed at the mucosal surface and not systemically

(Lightowlers, 1994). It should be noted that covert antigen preparations are successful because the target parasite ingests host blood and, therefore, systemic antibody. The effectiveness of this approach is less clear for non-blood feeding nematodes such as *Trichostrongylus* spp.. Recombinant vaccines, such as the *B. microplus* vaccine, which do not immediately eliminate the problem parasite, but steadily reduce numbers after several generations due to reduced fecundity, may be more difficult to introduce to farmers (Lightowlers, 1994). The final vaccine product against GI nematodes will probably consist of 3-5 parasite antigens and be effective towards several spp. (Emery, McClure and Wagland, 1993). Vaccination will most likely not be completely protective (sterile immunity) but will allow maintenance of a low manageable field level of infection to help extend and stabilise immunity within the flock (Emery, McClure and Wagland, 1993).

1.16 OBJECTIVES OF THE RESEARCH DESCRIBED IN THIS THESIS

At the time of commencing this study, there was little information on the nature of the ES components of *T. vitrinus*. The research carried out in this thesis aimed to characterise some of the components excreted and secreted *in vitro* by adult *T. vitrinus*, both by biochemical and molecular biological analysis. At the beginning of the study there was four principal objectives:

(i) To partially define the biochemical nature of *T. vitrinus* ES by the detection and definition of AChE and proteinases.

(ii) To isolate adult *T. vitrinus* cDNA fragments encoding AChE(s) using the polymerase chain reaction and degenerate oligonucleotide primers directed against highly conserved regions of the AChE molecule.

iii) To isolate putative ES components by the immunoscreening an adult *T. vitrinus* cDNA lambda gt11 library with antiserum raised against adult *T. vitrinus* ES and to define the proteins encoded by selected immunopositive clones by sequence analysis and database searches.

(iv) To further characterise the cDNA insert(s) in selected immunopositive clone(s) by Northern and Southern blotting and by the isolating full-length coding sequence.

Chapter two

Materials and Methods

2.1 CHEMICAL, ENZYME AND ISOTOPE SUPPLIERS

General laboratory chemicals (analar or molecular biology grade) were purchased from:

British Drug House Limited (BDH), Poole, Dorset, UK.

Difco, East Molesey, Surrey, UK.

Fisons Scientific Equipment, Leicestershire, UK.

Pharmacia Fine Chemicals, Uppsala, Sweden.

Sigma Chemical Company Limited, Poole, Dorset, UK.

Ultrapure agarose was obtained from Northumbria Biologicals Limited, Nelson Industrial Estate, Cramlington, Northumberland, UK.

Enzymes were supplied by Boehringer Mannheim UK (Diagnostics and Biochemicals) Limited, East Sussex, UK.

Stabilised aqueous solutions of 'redivue' α-^{32}P-dCTP (110 TBq/mmol; 370 MBq/ml) and α-^{35}S-dATP (220 TBq/mmol; 370 MBq/ml) were purchased from Amersham International plc., Buckinghamshire, UK.

2.2 STRAINS

2.2.1 *Trichostrongylus vitrinus* nematode

T. vitrinus used throughout this study was kindly provided by Dr. F Jackson and his staff, Moredun Research Institute, Edinburgh, UK. The parasite strain had been maintained at the Moredun Research Institute over a number of years by repeated passage through donor lambs maintained worm-free from birth.

2.2.2 Host *E.coli* strains

All host *E. coli* strains are listed in table 2.1.

Table 2.1 Host *E. coli* strains.

E. coli strain	Genotype	Notes
INVαF'	F', *end*A1, *rec*A1, *hsd*R17(r_k^-, m_k^+), *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1, ϕ80lacZαΔM15 Δ(lacZYA-argF)U169, deoR+	Supports the growth of pCR 1000 and pCR II plasmid vectors (Invitrogen, unpublished data).
JM109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, λ$^-$, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lacI*^{q}ZΔM15]	Supports the growth of vectors such as pBluescript II SK (+) plasmid vector (Yanisch-Perron, Vieira and Messing, 1985).
Y1090	*hsd*($r_k^-m_k^+$), Δ*lac*U169, *pro*A$^+$, Δ*lon*, *ara*D139, *str*A, *sup*F [*trp*C22::Tn*10* (*tet*^{r})] (pMC9)	Host strain used for propagation and expression of λgt11 cDNA library for immunoscreening (Young and Davis, 1983 a and b).
Y1089	*hsd*($r_k^-m_k^+$), Δ*lac*U169, *pro*A$^+$, Δ*lon*, *ara*D139, *str*A, *hfl*A150 [chr::Tn*10* (*tet*^{r})] (pMC9)	Host strain used for producing recombinant lysogens, thereby permitting larger quantities of the fusion peptide to be produced (Young and Davis, 1983 a and b).
MC1061	F$^-$, *hsd*R, *mcr*B, *ara*D139, Δ(*araABC-leu*)7679, *gal*U, *gal*K, Δ(*lac*)X74, *rps*L, *thi*	Host strain used for primary λSHLX2 cDNA library (Meissner, Sisk and Berman, 1987).

2.2.3 Plasmids

(i) pCR 1000 and pCR II plasmid vectors

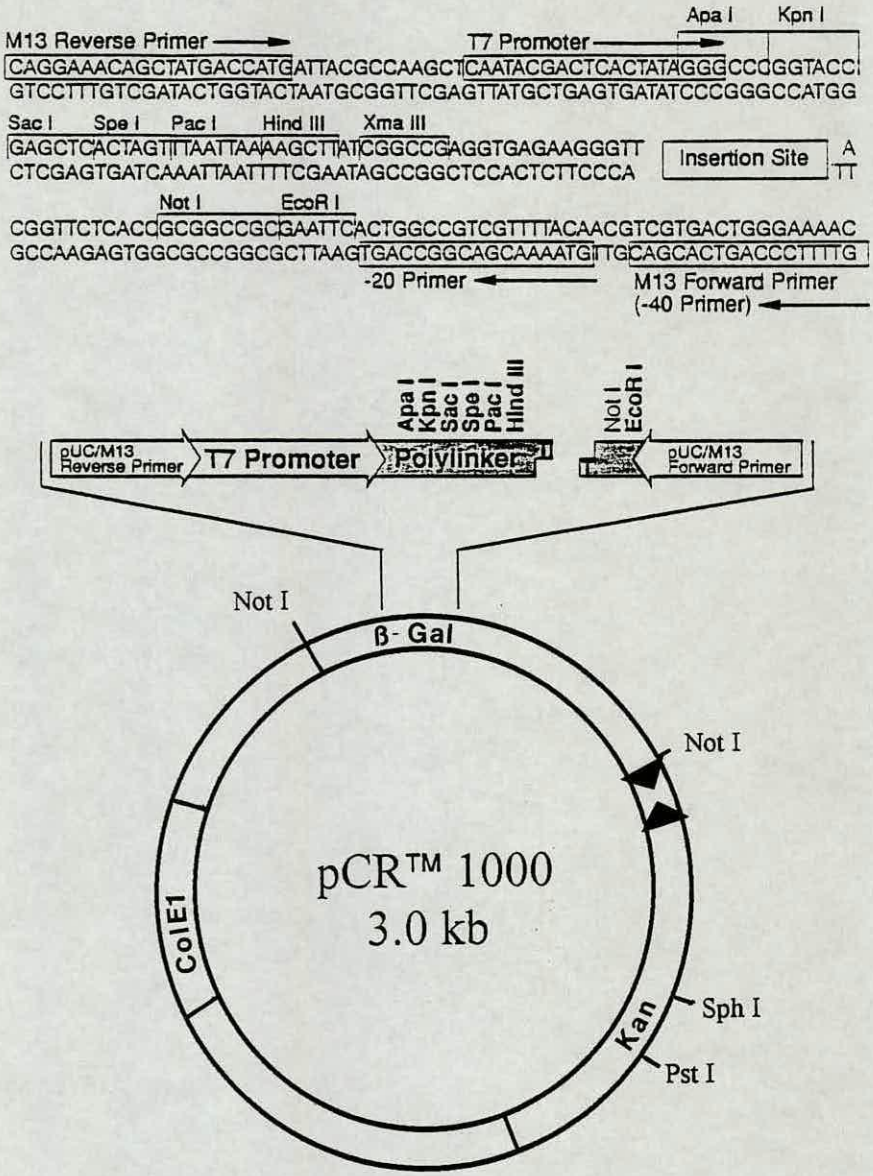
The pCR 1000 plasmid vector (unpublished data; Invitrogen, The Netherlands) is designed for subcloning of polymerase chain reaction (PCR) DNA templates directly, utilising the single deoxyadenosines added to the 3'-end of the PCR amplified product by the non-template dependant activity of *Taq* polymerase. pCR 1000 (figure 2.1; unpublished data; Invitrogen, The Netherlands) has recently been superseded by the pCR II plasmid vector (figure 2.2; unpublished data; Invitrogen, The Netherlands). pCR 1000 and pCR II plasmids containing no recombinant DNA inserts, grow as blue colonies on hosts harbouring the lacZΔM15 genotype in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Isopropylthio-β-D-galactoside (IPTG) is not required as cells of the *E. coli* host strain, IFNαF', are $LacI^{q}$. pCR 1000 and pCR II harbouring recombinant DNA inserts grow as white colonies as they are unable to utilise X-gal.

(ii) pBluescript II SK (+) plasmid vector

The pBluescript II SK M13 (+) plasmid vector (Short *et al.*, 1988) contains the M13 origin of replication and a polylinker with 21 unique restriction enzyme recognition sites for insertion of foreign DNA (figure 2.3). The polylinker is flanked by T3 and T7 RNA polymerase promoters which have been introduced into the N-terminal region of the *lacZ* gene allowing the identification of recombinant plasmids in a suitable host such as JM109, on the basis of blue/white selection as similarly described for the pCR 1000 and pCR II vectors. An ampicillin resistance gene provides a means of selecting for transformants in *E. coli*.

Figure 2.1

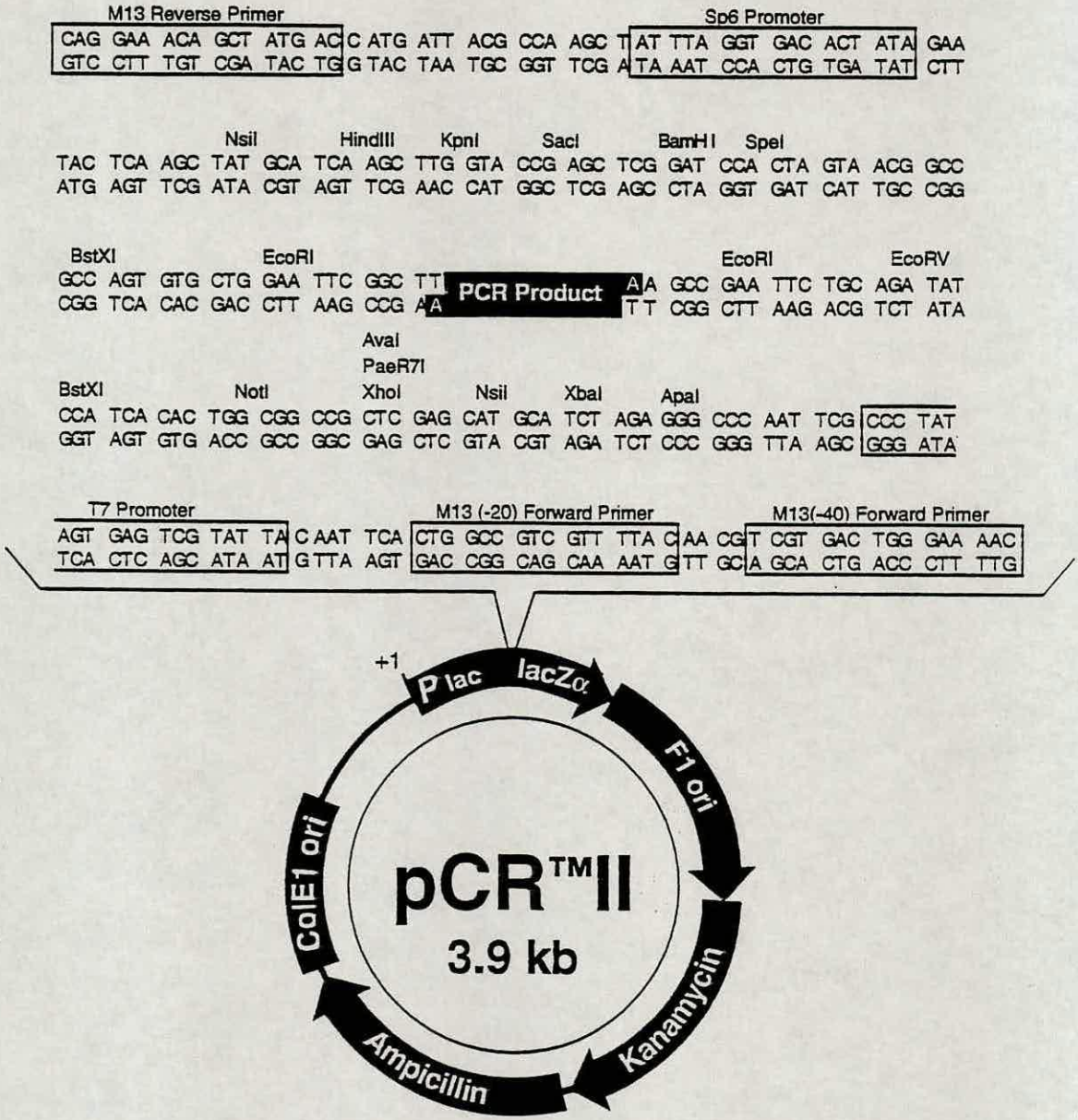
Map of the multiple cloning site for the linearised pCR 1000 plasmid vector.



The pCR 1000 plasmid vector is 3.0 kb in size. The restriction endonuclease cleavage sites and the positions of the M13 primer sequences and Sp6 and T7 promoter sequences within the multiple cloning site (MCS) in the *lacZ* gene are shown. The linearised vector has single 3' deoxythymidine residues, allowing PCR products (with single deoxyadenosine 3' ends added by *Taq* polymerase) to be ligated efficiently with the vector. Other features of the vector include a ColE1 origin and a kanamycin (Kan) resistance gene. The figure was adapted from the Invitrogen 'TA cloning kit' instructions (version 1.1).

Figure 2.2

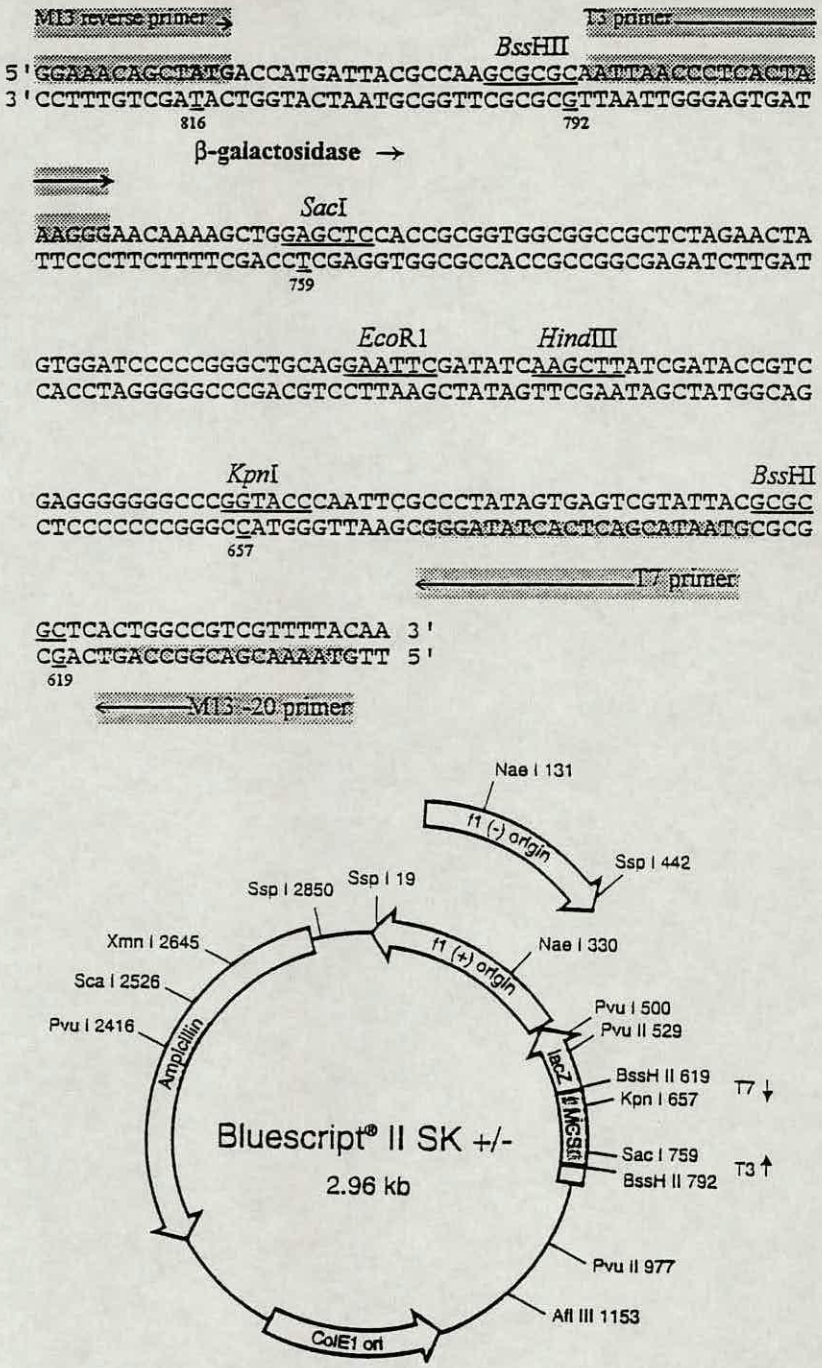
Map of the multiple cloning site for the linearised pCR II plasmid vector.



The pCR II plasmid vector is 3.9 kb in size. The restriction endonuclease cleavage sites and the positions of the M13 primer sequences and Sp6 and T7 promoter sequences within the MCS in the *lacZ* gene are shown. The linearised vector has single 3' deoxythymidine residues, allowing PCR products (with single deoxyadenosine 3' ends added by *Taq* polymerase) to be ligated efficiently with the vector. The inserted PCR product is flanked on each side by *Eco*RI sites. Other features of the vector include a ColE1 origin, f1 origin and ampicillin and kanamycin resistance genes. The figure was adapted from the Invitrogen 'TA cloning kit' instructions (version 1.3).

Figure 2.3

Map of the multiple cloning site for the pBluescript II SK (+) plasmid vector.



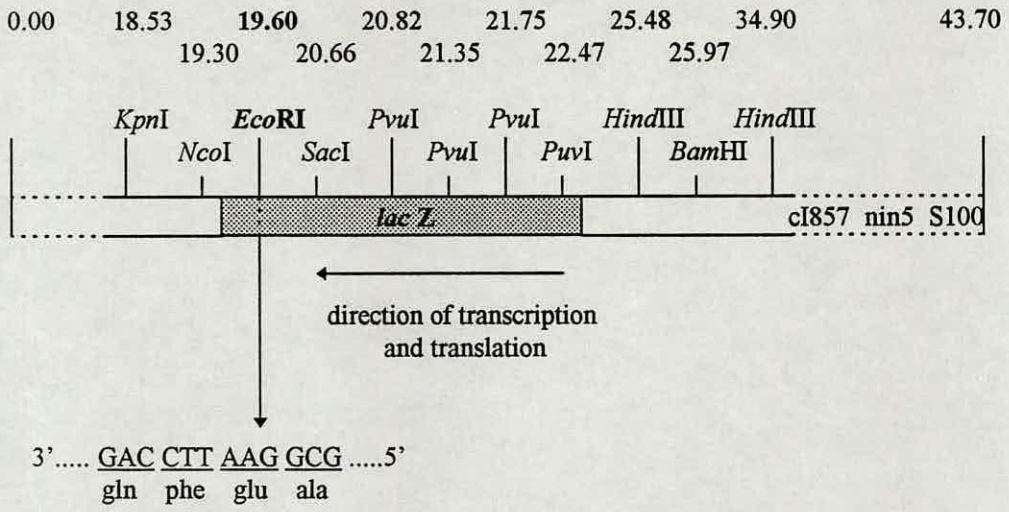
The pBluescript II SK (+) plasmid vector is 2.96 kb in size and is derived from pUC19. The important restriction endonuclease cleavage sites and the positions of the M13 forward and reverse primer sequences and T3 and T7 promoter sequences within the MCS in the *lacZ* gene are shown. Other features of the plasmid include ColE1 origin, f1 origin and an ampicillin resistance gene. The designation, SK, is given as the polylinker is orientated so that *lacZ* transcription proceeds from *Sac*I to *Kpn*I. The figure was adapted from Stratagene's catalogue 1994.

2.2.4 Bacteriophage lambda gt 11 (λgt11)

The bacteriophage vector, λgt11, is used for the in-frame expression of foreign fragments, usually cDNA (Young and Davis, 1983 b). A map of λgt11 is shown in figure 2.4. Foreign DNA (up to 7.2 kb) is inserted into a unique *EcoR1* restriction site located within the *lacZ* gene, 53 bp upstream from the β-galactosidase translation termination codon. This enables in-frame inserted DNA to be expressed as fusion proteins with β-galactosidase and recombinants of interest can be selected using appropriate specific antisera. In addition, insertion of foreign DNA into *lacZ* results in the inactivation of β-galactosidase and recombinant phage can be selected by blue/white selection. The presence of a temperature-sensitive repressor (*c*I857) controls bacteriophage replication and production of the fusion protein: inactivation of the *c*I857 gene product at 43°C promotes the lytic cycle and plaque formation; lysgeny occurs at lower temperatures. Also, the phage encodes an amber mutation (*S*100) that renders it lysis-defective in hosts which lack the amber suppressor *supF* (Young and Davis, 1983 b).

Figure 2.4

Schematic diagram of the bacteriophage lambda gt11.



Selected restriction endonuclease cleavage sites in and flanking the *Eco*RI site (shown in bold) within the *lac*Z gene are marked with distances in kilobase pairs from left to right. The nucleotide sequence of the *Eco*RI site and the amino acid residues for which encodes are also shown. The positions of *c*I857, nin5 and *S*100, in relation to the *lac*Z gene are indicated. The figure was adapted from Amersham's 'cDNA cloning system λgt11' kit instruction manual.

2.3 OTHER MATERIALS

Filter paper used was Whatman number 3 (3 MM), supplied by Whatman Limited, Maidstone, Kent, UK.

Hybond™-N nylon transfer membrane (0.45 micron pore) for Northern and Southern blotting was obtained from Amersham.

Hybond™-N nylon transfer membrane (0.45 micron pore) used for plaque lifts in immunoscreening was also obtained from Amersham.

Immobilon-P (0.45 μm) transfer membrane for Western blotting was purchased from Millipore Corporation, Bedford, UK.

RX medical X-ray film was obtained from Fuji, Japan.

X-ray film developer (CDL8) and fix (CF40) were purchased from Photosol Ltd, Basildon, UK.

MSE (Loughborough, Leistershire, UK) micro centaur microfuge and MSE Mistral 2000 centrifuges were used throughout.

2.4 SOLUTIONS

Standard solutions were made with deionised distilled water, unless otherwise stated, and where indicated, were sterilised by autoclaving at 15 psi/15 min. All glassware was sterilized by this procedure before use.

2.4.1 General solutions

Phenol

Redistilled phenol (stored at -20°C) was melted at 68°C, cooled to room temperature and hydroxyquinoline was added to a final concentration of 0.1% (w/v). After equilibriation with several changes of 100x TE buffer concentrate, pH 8.0 (see below), the final aqueous layer was removed and 0.1 vol of 1x TE buffer (pH 8.0) containing 0.2% β-mercaptoethanol, was added. The phenol solution was stored in a light tight bottle at 4°C.

Phenol:chloroform

Phenol was mixed 6:4 (v/v) with chloroform:isoamylalcohol (24:1, v/v) and stored in a dark bottle at room temperature.

Chloroform:isoamylalcohol

Chloroform was mixed with isoamylalcohol (24:1, v/v) and stored at room temperature in a dark bottle.

30% acrylamide stock for PAGE

An aqueous solution, containing 29.2% (w/v) acrylamide and 0.8% (w/v) bis acrylamide was prepared.

40% acrylamide stock for sequencing gel

An aqueous solution, containing 38% (w/v) acrylamide and 2% (w/v) bis acrylamide was prepared.

RNA extraction buffer

A solution, containing 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% (w/v) N-lauryl sarcosine and 0.7% (v/v) 2-β-mercaptoethanol was prepared in sterile distilled water.

DNA extraction buffer

50 mM Tris-HCl buffer, pH 7.5 (see below) was prepared, containing 100 mM sodium chloride (NaCl), 1 mM disodium ethylenediaminotetraacetate (Na_2EDTA), 1% (w/v) sodium dodecyl sulphate (SDS) and 200 μg/ml proteinase K.

Tris-EDTA buffer (TE)

10 mM Tris-HCl buffer, pH 8.0 (see below) was prepared, containing 1 mM Na_2EDTA (pH 8.0) and was sterilised by autoclaving.

Tris buffered saline (TBS)

50 mM Tris-HCl buffer, pH 8.0 (see below) was prepared, containing 150 mM NaCl.

TBS with Tween (TBST)

TBST was prepared by adding 0.5 ml of Tween-20 to 1 litre of TBS.

20x sodium chloride-citrate (SSC)

A solution containing 3.0 M NaCl and 300 mM tri-sodium citrate was prepared in distilled water and was adjusted to pH 7.0 with 5 M sodium hydroxide (NaOH).

SM buffer

An aqueous solution containing 0.1 M NaCl, 1 mM magnesium sulphate ($MgSO_4$), 50 mM Tris base and 0.1% (v/v) gelatin was prepared and was adjusted to pH 7.5 with hydrochloric acid (HCl). The solution was sterilised by autoclaving.

Phosphate buffered solution (PBS)

A solution of 0.1 M phosphate buffer, pH 7.4 (see below) was prepared containing 0.9% (w/v) NaCl and was sterilised by autoclaving.

Sodium acetate

An aqueous solution of 3 M sodium acetate was adjusted to pH 5.2 with glacial acetic acid and was sterilised by autoclaving .

Ammonium acetate

An aqueous solution of 4 M ammonium acetate was adjusted to pH 6.4 with HCl and was sterilised by filtration (0.45 micron pore discs; Amicon Ltd., Stonehouse, Gloustershire, UK).

Na_2EDTA

A 0.5 M solution of Na_2EDTA was adjusted to pH 8.0 with NaOH and was sterilised by autoclaving.

X-gal

A stock solution of X-gal was prepared by dissolving 40 mg of X-gal in 1 ml of dimethylformamide. The solution was stored at -20°C.

IPTG

A stock solution of IPTG was prepared by dissolving 40 mg of IPTG in 1 ml of deionised distilled water. This was subsequently filter sterilised (0.45 micron pore filter) and stored at -20°C.

Denhardt's solution (100x stock)

A solution containing 2% (w/v) Ficoll (type 400), 2% (w/v) polyvinylpyrolidine, 2% (w/v) bovine serum albumin (BSA, Fraction V) was prepared in sterile distilled water.

Diaminobenzidine tetra-hydrochloride (DAB) substrate for immunodetection

DAB (50 mg) was dissolved in 5 ml methanol, made up to 50 ml with TBS and 100 µl of 30% hydrogen peroxide was added.

2.4.2 Electrophoresis buffers

Tris-acetate (TAE) buffer

A 50x concentrated stock of TAE buffer was prepared by dissolving 242 gm of Tris base in 500 ml of deionised distilled water. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M Na_2EDTA (pH 8.0) was added and the solution was made up to 1 litre with more deionised distilled water. The final working 1x TAE buffer contained 40 mM Tris-acetate and 2 mM Na_2EDTA and was sterilised by autoclaving.

Tris-borate (TBE) buffer

A 10x concentrated stock solution of TBE buffer was prepared by dissolving 121 gm of Tris base, 53.4 gm of boric acid and 7.4 gm of Na_2EDTA in deionised distilled water (the final vol was 1 litre). The final working 1x TBE buffer contained 90 mM Tris-borate and 1 mM Na_2EDTA and was sterilised by autoclaving.

Laemmli buffer

An aqueous solution, containing 25 mM Tris base, 250 mM glycine and 0.1% (w/v) SDS was prepared (Laemmli, 1970). The buffer was made as a 5x concentrated solution.

Loening "E" buffer

An aqueous solution, containing 36 mM Tris base, 30 M sodium dihydrogen phosphate and 1 mM Na_2EDTA was prepared. The buffer was made as a 5x concentrated solution.

MOPS/EDTA buffer

An aqueous solution, containing 20 mM MOPS [3-(morpholino) propanesulphonic acid], pH 7.0, 5 mM sodium acetate and 1 mM Na_2EDTA was prepared. The buffer was made as a 10x concentration stock.

Sample buffer for agarose gel electrophoresis & DNA polyacrylamide gel electrophoresis (DNA-PAGE)

An aqueous solution containing 0.25% (w/v) bromophenol blue, 40% (w/v) sucrose and 1 mM Na_2EDTA was prepared.

Sample buffer for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

(a) Non-reducing buffer: 62.5 mM Tris-HCl buffer, pH 6.8 (see below) was prepared containing 10% (v/v) glycerol, 2% (w/v) SDS and 0.00125% (w/v) bromophenol blue.

(b) Reducing buffer: the buffer was prepared as described in (a), with the addition of 5% (v/v) β-mercaptoethanol.

2.4.3 pH buffers

All pH buffers were sterilised by autoclaving.

pH 3.0-6.0 sodium acetate buffers

An aqueous solution, containing 0.1 M sodium acetate was prepared and adjusted to the correct pH with glacial acetic acid.

pH 5.5-8.0 phosphate buffers

Phosphate buffer was prepared by mixing the required quantities of 0.1 M sodium hydrogen orthophosphate (Na_2HPO_4) with 0.1 M potassium dihydrogen orthophosphate (KH_2PO_4) to give the desired pH.

pH 7.5-9.5 Tris buffers

An aqueous solution, containing the desired molarity of Tris base, was prepared and adjusted to the required pH with HCl.

pH 9.5-11.0 sodium carbonate-bicarbonate buffers

Sodium carbonate-bicarbonate buffer was prepared by mixing 0.1 M sodium carbonate (Na_2CO_3) with 0.1 M sodium bicarbonate ($NaHCO_3$) to give the required pH.

2.4.4 Media

LB-broth

Bactotryptone (10 gm), bacto yeast extract (5 gm), NaCl (10 gm) were dissolved in 800 ml of deionised distilled water. The solution was adjusted to pH 7 with HCl and the volume was increased to 1 litre with water. The LB-broth was then sterilised by autoclaving at 15 psi for 20 min.

LB-agar plates

Prior to autoclaving, 15 gm/l of bacto-agar was added to LB-broth. After cooling to 43°C, the required antibiotic was added and approximately 25 ml was poured into 90 mm diameter sterile petri dishes (Bibby Sterilin Ltd, Staffordshire, UK) and left to cool on a level surface until the agar had solidified. The agar plates were stored at 4°C and, before use, they were dried briefly at 37°C.

Top agar

Bacto agar (0.7 gm/100 ml) was added to the LB-broth prior to autoclaving.

LB-agarose & top agarose

For LB-agarose and top agarose, agarose (0.7 gm/100 ml) was substituted for the bacto agar in the recipes above.

SOC medium

An aqueous solution, containing 2% (w/v) bactotryptone, 0.5% (w/v) bactoyeast extract, 10 mM NaCl, 2.5 mM potassium chloride (KCl), 10 mM magnesium chloride ($MgCl_2$), 10 mM $MgSO_4$ and 20 mM glucose was prepared and was then sterilised by autoclaving.

2.4.5 Antibiotics

Ampicillin

A concentrated stock of 50 mg ampicillin dissolved in 1 ml of deionised distilled water was filter sterilised (0.45 micron filter) before storage at -20°C.

Kanamycin

A concentrated stock of 10 mg kanamycin per ml of deionised distilled water was filter sterilised through a 0.45 micron filter prior to storage at -20°C.

Tetracycline

A concentrated stock of 5 mg of tetracycline, dissolved in 1 ml of ethanol and was stored at -20°C.

2.4.6 Proteinase inhibitors

Phenylmethylsulphonyl flouride (PmsF)

A concentrated stock solution of 50 mM PmsF in methanol was prepared.

Trans-*epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E64)*

A concentrated stock of an aqueous solution containing 800 μM E64 was prepared.

Pepstatin

A concentrated stock of 40 μM pepstatin in methanol was prepared.

2.5 ANTISERA

Rabbit anti-adult *T. vitrinus* excretory-sectertory (ES) serum was obtained as described in section 2.8.7.

Normal rabbit serum was purchased from Sigma.

Serum from sheep immune to *T. vitrinus* infection and pre-immune sheep serum were kindly provided by Dr. F. Jackson, Moredun Research Institute, Edinburgh, UK.

Rabbit anti-*T. colubriformis* secretory AChE serum was a kind gift from G. Griffiths, University of Nottingham, Nottingham, UK.

Guinea-pig anti-rabbit IgG serum conjugated to horseradish peroxidase was purchased from Sigma.

Donkey anti-sheep IgG serum conjugated to horseradish peroxidase was obtained from the Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, UK.

2.6 OLIGONUCLEOTIDE PRIMERS

Oligonucleotide primers were synthesised by Oswel DNA Services, University of Edinburgh, Edinburgh, UK, unless otherwise stated. The general oligonucleotides used are outlined in table 2.2.

2.7 *CAENORHABDITIS ELEGANS (C. ELEGANS)* CHOLINESTERASE CLONES

C. elegans cDNA fragments encoding cholinesterase-like peptides (clones cm 6b1, cm 7d7 & cm 10g6, Waterston *et al.*, 1992) were a kind gift from Dr. A. Coulson, MRC Laboratory of Molecular Biology, Cambridge, UK. Further information on each of the clones may be found in section 2.10.15 and chapter 4.

Table 2.2 General oligonucleotide primers.

Primer (sense/ antisense)	**Oligonucleotide primer sequence** 5' → 3'	**Notes**
dT (antisense)	*Eco*R1 site 5'ACA GAA TTC TTT TTT TTT TTT TTT 3'	Directed towards the poly A^+ tail of mRNA to allow amplification of the 3' region of the gene. Includes an *Eco*RI restriction enzyme site to facilitate cloning.
SL 1 (sense)	5' GGT TTA ATT ACC CAA GTT TGA G 3'	Nematode conserved splice leader 1 sequence, present 5' to start codon in many nematode genes (reviewed by Nilsen, 1993).
514N (sense)	5' GGT GGC GAC GAC TCC TGG AGC CCG 3'	Directed towards λgt11 vector arm 37 to 14 bp 5' from *EcoR1* site (Moran *et al.*, 1990).
515N (antisense)	5' TTG ACA CCA GAC CAA CTG GTA ATG 3'	Directed towards λgt11 vector arm 23 to 55 bp 3' from *EcoR1* site (Moran *et al.*, 1990).
G0507 (sense)	5' CAT CGC CAT CTG CTG CAC GCG GAA 3'	Directed towards λgt11 vector arm 103 to 77 bp 5' from *EcoR1* site (Moran *et al.*, 1990).
G0508 (antisense)	5' GAG GAT ACG TTT CAC TAT GAG AGC 3'	Directed towards λgt11 vector arm 214 to 237 bp 3' from *EcoR1* site (Moran *et al.*, 1990).
SP6 promoter (antisense)	5' ATA GAA TAT GCA TCA AGC TGA G 3'	Directed towards λSHLX vector arm, -2 to +20 bp 3' to the *Sac1* site (Palazzola *et al.*, 1990).
T7 promoter (sense)	5' ACT ATA GGG AGC TAA GCT TGG 3'	Directed towards λSHLX vector arm, 24 to 4 bp 5' to the *Apa1* site (Palazzola *et al.*, 1990).

Table 2.2 cont.

Primer (sense/ antisense)	**Oligonucleotide primer sequence** 5' → 3'	**Notes**
M13 (-20) forward primer (antisense)	5' GTA AAA CGA CGG CCA GT 3'	Bluescript and pCR II plasmid vectors both contain the bacteriophage M13 origin of replication. The M13 forward and reverse primers can be used for sequencing or PCR amplification across recombinant DNA insertion site (Short *et al.*, 1988).
M13 reverse primer (sense)	5' GGA AAC AGC TAT GAC CAT G 3'	
λgt11 adapter primer (sense)	*Eco*RI *Bam*HI 5' AA TTC GAG GAT CCG phe glu asp pro *Kpn*I *Nco*I GGT ACC ATG G 3' gly thr met	*Eco*RI adapter primer provided with Amersham's 'cDNA cloning system λgt11' kit. The primer carries a blunt end for ligating cDNA and an *Eco*RI cohesive end for subsequent ligation with λgt11 arms. The primer contains *Bam*HI, *Kpn*I and *Nco*I sites. The in-frame amino acid sequence for the primer is also shown (Amersham).

2.8 PARASITOLOGICAL TECHNIQUES

2.8.1 *T. vitrinus* nematode

T. vitrinus nematode worms were kindly prepared by Dr. F. Jackson, Moredun Research Institute, Edinburgh, UK. As required, 8 month old lambs were infected with 300,000 infective L3 from a pure isolate of *T. vitrinus*, as detailed in section 2.2.1. At 7 or 14 days p.i. the lamb was killed in order to harvest L4 or adult worms respectively. The proximal small intestine was removed, slit longitudinally and most of the food particles were removed from the surface by gently rinsing the intestine with warm water. The intestine was then placed in saline for 4 hr at 37°C, during which time, the parasites had migrated out of the intestinal mucosa. At this stage, the parasites were contained in 50 ml of saline. The parasites were washed as described in the next section.

2.8.2 Washing of parasites

Following the harvesting of the *T. vitrinus* worms from the small intestine (as described above), there were still some food particles contaminating the culture. These were removed by a series of washes in sucrose solutions.

A 25% sucrose solution (100 ml) was added to the parasite suspension. Following centrifugation at 300 g for 10 min the supernatant (parasites) was removed to a separate container. The pellet (mostly food particles) was resuspended in a further 10 ml 25% sucrose and the sample centrifuged as before. Again, the supernatant was removed and pooled with the previous supernatant (the pellet was discarded). To enable the parasites to congregate at the bottom of the supernatant, 4 vols of 10% sucrose were added and the parasite suspension was left to stand at room temperature for 30 min. The excess sucrose solution was removed and the parasites were washed with 50 ml sterile PBS (section 2.4.1). Following this, the parasites were allowed to settle at the bottom of the PBS solution for 30 min before the PBS was removed by aspiration.

2.8.3 Storage of parasite material

The parasites were blotted with a tissue to remove excess fluid, weighed and placed in sterile eppendorfs for storage in liquid nitrogen.

2.8.4 Parasite culture proceeding to harvest of excretory-secretory material (ES)

Adult worms (500/ml) or L4 larvae (1000/ml) were cultured overnight in sterile PBS containing streptomycin (50 μg/ml), penicillin (0.5 units/ml) and gentamicin (125 μg/ml), with constant, gentle shaking using a shaking waterbath at 37°C. The culture fluid (ES) was removed and centrifuged at 300 g for 5 min to sediment any contaminating worms. The ES was transferred to sterile tubes and was stored in suitable aliquots at -70°C until required. Viability of the parasites at the end of the culture period was confirmed by microscopic examination. Aliquots were streaked onto LB-agar plates in the absence of antibiotics and the plates were incubated overnight at 37°C to check for bacterial contamination.

2.8.5 Concentration of ES material

ES material was concentrated 40-80 fold, using centriprep 10 or centricon 10 microconcentrators (Amicon Ltd., Stonehouse, UK) which concentrate proteins above 10 kDa, by centrifugation, usually for 1 hr at 1,500 g using a MSE Mistral 6L centrifuge set at 4°C.

2.8.6 Preparation of adult *T. vitrinus* homogenate

Adult *T. vitrinus* worms (0.5 gm wet weight, stored in liquid nitrogen) were powdered using a sterile, prechilled (-70°C) pestle and mortar and were solubilised in 5 ml of sterile PBS (section 2.4.1). The solution was centrifuged at 5,000 g for 5 min after which, the supernatant was removed and centrifuged at 100,000 g for 1 hr (Beckman TL 100 ultracentrifuge; Beckman, High Wykham, UK). The supernatant was removed and stored at -70°C.

2.8.7 Rabbit anti-adult *T. vitrinus* ES serum

Approximately 200 μg of concentrated ES protein (protein concentration determined as detailed in section 2.9.1) in 1 ml PBS was emulsified with 1 ml Freund's Complete Adjuvant and injected subcutaneously, in 0.5 ml aliqouts, along the back of the rabbit. At 5 and 8 weeks post initial dose, the rabbit was boosted with 200 μg of concentrated ES protein, emulsified in Freund's Incomplete Adjuvant in the same way. Serum was collected at 2 and 4 weeks after the final boost and the antibody response was monitored by Western blotting.

2.9 PROTEIN AND ENZYME CHARACTERISATION

Section A: Protein characterisation

2.9.1 Protein determination by spectrophotomeric assay

The protein content of *T. vitrinus* homogenate and ES material was determined using the bicinchonic acid (BCA) protein assay kit from Pierce, Rockford, IL, USA (based on the method of Smith *et al.*, 1985). In an alkaline medium, protein reacts with Cu^{2+} to produce Cu^{+}, this in turn reacts with BCA. The resulting purple coloured product is water soluble and may be measured by absorption at 562 nm.

The "Working BCA Protein Assay Reagent" was prepared by mixing Reagent A (BCA solution):Reagent B (4% $CuSO_4$ solution) in a ratio of 50:1. A set of protein standards (0.25 μg - 50 μg/ml) were made by diluting a stock solution of BSA in water. An aliquot of the test sample or standard (100 μl) was combined with 2 ml of the "Working Reagent" and incubated at 37°C for 30 min. After incubation, the samples were cooled to room temperature and the absorbance measured at 562 nm in a Cecil 595 UV/visible spectrophotometer. The samples were referenced to a water blank negative control. The absorbance of the blank was subtracted from the value of the standards and test samples. A standard curve was plotted of the net absorbance of the standards at 562 nm against protein concentration and, using this standard curve, the protein concentration for each test sample was determined. A micro-protocol was also developed using 1/10 of the volumes described above and the absorbances (562

nm) were determined using a Monarch microcentrifugal analyser (Instrumentation Laboratory, Warrington, UK).

2.9.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiles were routinuely analysed electrophophoretically by SDS-PAGE, carried out using a method based on that of Laemmli (1970). The highly cross-linked polyacrylamide provides an inert matrix through which the proteins migrate according to their molecular size. SDS is an an anionic detergent that disrupts nearly all non-covalent interactions in native proteins. Inclusion of β-mercaptoethanol in the loading buffer reduces disulphide bonds within the proteins.

Resolving gels (to give a final concentraton of 5.0-10% acrylamide [w/v] in buffer, containing 0.1% [w/v] SDS) were prepared by combining the appropriate vol. of 4x resolving buffer (1.5 M Tris-HCl, pH 8.8), 30% acrylaminde stock solution (section 2.4.1), 10% (w/v, in distilled water) SDS and distilled water. Ammonium persulphate and N,N,N',N'-tetrametylethylenediamine (TEMED) were added to a final concentration of 0.05% (w/v) and (v/v) respectively, immediately before casting the gel. Stacking gels (at a final concentration of 4% acrylamide [w/v] in buffer solution) were prepared in the same way, except that 4x stacking gel buffer (0.5 M Tris-HCl, pH 6.8) was substituted for the 4x resolving gel buffer. Gels were run using Laemmli Buffer (see section 2.4.2) in the "Mini-Protean" gel system (Bio-rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK) at a constant voltage of 200 V for 45 min. Before being loaded, an equal vol of SDS-PAGE Reducing Sample Buffer (section 2.4.2) was mixed with the sample and then boiled for 5 min. High molecular weight protein markers (205, 116, 97, 66, 45, 29 kDa; Sigma) were co-electrophoresed to estimate the size of the separated proteins.

Samples to be analysed for acetylcholinesterase and esterase were run under native conditions such that SDS and β-mercaptoethanol were excluded from all the buffers and the samples were not boiled, as these treatments would disrupt enzyme protein structure inhibiting enzyme activity.

2.9.3 Protein stains for samples analysed by PAGE

Coomassie blue stain

Polyacrylamide gels were stained for protein using Coomassie Brilliant Blue. After electrophoresis, the gel was immersed in 0.1% (w/v) Coomassie Brilliant Blue R250 dissolved in methanol/acetic acid/water solution in a ratio of 3:1:6 respectively, for 30 min on a shaker at room temperature. The dye was removed and the gel destained in the methanol/acetic acid/water solution alone. Protein bands appear as dark blue bands on a clear background.

Silver stain

As a more sensitive method for detecting protein bands on a polyacrylamide gel, the Bio-Rad Silver Stain kit was used which was based on the method of Gottlieb and Chavko (1987). Included in the kit were the 'oxidiser', 'silver reagent' and 'developer', the chemical compositions of which were not given. The gel was fixed in methanol/acetic acid/water solution (4:1:6 v/v/v respectively) for 30 min initially, followed by an ethanol/acetic acid/water solution (1:0.5:8.5 v/v/v respectively) for two periods of 10 min each. Once fixed, the gel was immersed in 'oxidiser' solution for 3 min and then repeatedly washed in distilled water until the yellow colour was removed from the gel. After incubation in the 'silver reagent' for 15 min, the gel was immersed in 'developer' solution for <1 min. This was removed, and fresh 'developer' was added. Protein bands appeared dark brown against a pale background. Once bands had been developed to the desired intensity the reaction was stopped by placing the gel in 5% acetic acid solution (v/v in distilled water).

2.9.4 Western Blotting and immunodetection of proteins

(i) transfer of proteins to membrane

Proteins were transferred from polyacrylamide gels to 'immobilon' polyvinylidene diflouride transfer membrane using a Mini-Protean Transblot System (Biorad) according to the manufacturer's instructions as modified from the method of

Towbin, Staehlin and Gordon (1979). Before 'immobilon' can be moistened with buffer it must first be soaked for 2 min in methanol and then washed in distilled water.

After electrophoresis, the protein gels were equilibrated in blot transfer buffer (an aqueous solution containing, 25 mM Tris base and 192 mM glycine, pH 8.3) for 15 min to remove the SDS. A sandwich was assembled with the following successive layers: a porous fibre pad, a sheet of Whatman 3 MM filter paper, the polyacrylamide gel, the transfer membrane, a sheet of Whatman 3 MM filter paper and finally another porous pad. All components were pre-wetted with transfer buffer. The sandwich was placed in the electrophoresis tank with the membrane towards the anode and the tank was filled with chilled (4°C) transfer buffer. Proteins were transferred for 1 hr at a constant current of 150 mA. Upon completion, the membranes were cut into the appropriate strips ready for probing with the relevant sera. One of the strips was stained for protein with Coomassie blue, as described above. The post transfer gel was also Coomassie stained to confirm transfer of the proteins to the membrane.

(ii) periodate treatment of Western blots

To cleave carbohydrate vicinal hydroxyl groups from proteins, without altering the polypeptide structure, the Western blots were treated with sodium periodate before the blots were blocked (Woodward, Young and Bloodgood, 1985). The blot was incubated (in the dark) with 50 mM sodium periodate solution, prepared in 50 mM sodium acetate buffer, pH 4.5, for 1 hour. The blots were then rinsed twice with the acetate buffer and once in TBS, before they were incubated with the blocking solution.

(iii) immunodetection of proteins on Western blots

All steps were carried out at room temperature with mild agitation of the sample solution. First, the membrane was blocked with low fat milk ('Marvel original' [Premier Beverages, Stafford, UK]; 5% w/v in TBST buffer) for 2 hr to reduce non-specific binding of antibodies. Following blocking, the membrane was washed (three, 10 min washes) in TBST (section 2.4.1) and incubated overnight with the primary antibody, diluted in TBS (section 2.4.1). The serum dilutions varied from

1:50 to 1:500 as indicated. After further washing in TBST, the membranes were incubated for 2 hr in the secondary antibody conjugated to horseradish peroxidase (HRP). The conjugate antibody was diluted 1:1000 in TBS. Finally, the membranes were washed again in TBST before adding a freshly prepared solution of the DAB substrate (section 2.4.1) for immunodetection. When the colour had developed to the desired intensity the substrate solution was removed by washing in distilled water and the membrane was air dried.

Section B: Acetylcholinesterase (AChE) and esterase characterisation

2.9.5 Spectrophotomeric assay for the detection of AChE activity

The AChE activity of *T. vitrinus* homogenate and ES material was determined by the colorimetric technique of Ellman *et al.* (1961), utilising acetylthiocholine iodide (ATCI) as the substrate. Hydrolysis of ATCI results in the production of thiocholine which reacts with a dithiobisnitrobenzoate ion to give a yellow coloured product.

The reaction solution was prepared by mixing the following volume of solutions: 750 µl of 0.1 M phosphate buffer (section 2.4.3), 5 µl of an aqueous solution, containing 75 mM ATCI and 25 µl of 0.1 M phosphate buffer pH 7.5, containing 10 mM 5'5'-dithiobi-2-nitrobenzoic acid (DTNB) and 18 mM sodium bicarbonate. The reaction solution was prewarmed to 37°C with a Tecam circulator. Sample (12.5 µl), or water for blanks, was added and the change in absorbance at 412 nm (at 37°C) was monitored for 5 min using a Cecil 595 UV/visible spectrophotometer connected to a Cecil 505 recorder. The test sample was corrected for non-enzymatic hydrolysis using the negative control reaction. Enzyme activity was expressed as international units (IU) of AChE activity, calculated as follows:

$$\text{IU AChE activity/l} = 4662 \times OD_{412}/\text{min}$$

Initially, the AChE activity was measured over a range of pHs to determine the optimal pH for activity. The phosphate buffer was replaced with the following buffers where appropriate: 0.1 M sodium acetate, pH 5.0-5.5; 0.1M phosphate, pH 5.25-8.0; 0.1 M Tris-HCl, pH 8.0-9.0 (see section 2.4.3).

2.9.6 PAGE analysis for esterase activity

Esterase activities of adult *T. vitrinus* homogenate and ES were visualised using native polyacrylamide gels by the method of Grunder, Sartori & Stormont (1965) which utilises α-napthyl acetate as the substrate. Liberated napthol reacts with Fast blue RR dye to yield an insoluble brown product.

After electophoresis the gel was incubated at room temperature in a solution of the following: 0.5 ml 2% (w/v) α-naphthyl acetate in acetone, 50 mg fast blue RR salt, 10 ml 0.1 M PBS (pH 7.0) and distilled water to 50 ml. Esterase activity appeared as dark brown bands on a light background.

2.9.7 PAGE analysis for acetylcholinesterase activity

AChE activity of adult *T. vitrinus* homogenate and ES was visualised on native polyacrylamide gels using the staining procedure developed by Karnovsky and Roots (1964). Hydrolysis of ATCI by AChE results in the production of thiocholine which reduces ferricyanide to ferrocyanide. The latter combines with Cu^{2+} to form insoluble copper ferrocyanate which is brown in colour. Citrate ions in the reaction mixture react with the Cu^{2+} ions to prevent them forming copper ferricyanide.

ATCI (25 mg) was dissolved in 32.5 ml of 0.1 M sodium acetate. To this was added (in given order with constant stirring) 2.5 ml 0.1 M sodium citrate, 5 ml 30 mM copper sulphate and 5 ml 5 mM potassium ferricyanide. Each of these solutions were prepared in deionised, distilled water. The gel was immersed in this solution at room temperature until the enzyme reaction was complete.

Section C: Proteinase characterisation

2.9.8 Spectrophotomeric assay for the determination of total proteinase activity

Determination of total proteinase activity was carried out essentially as described by Knox and Kennedy (1988). The substrate used was azocasein, a chromogenic derivative of casein where a yellow coloured azo dye is bound into the protein molecule. Hydrolysis of the protein liberates the soluble azo dye which can be measured in the supernatant following precipitation of undigested protein.

Total proteinase activity of adult *T. vitrinus* ES was measured using buffers in appropriate pH ranges to determine pH optima. These were as follows: 0.1 M sodium acetate, pH 4.0-6.0; 0.1 M phosphate buffer, pH 5.5-8.5; 0.1 M Tris-HCl, pH 8.0-9.5; 0.1 M carbonate/bicarbonate buffer, pH 9.5-10.5 (see section 2.4.3). An aqueous stock solution of 5 mg/ml of azocasein was prepared, 5 µl of which was mixed with 5 µl of sample, 50 µl of buffer and 2µl of penicillin (final concentration 0.5 units/ml)/streptomycin (final concentration 50 µg/ml) solution. For negative controls, the test sample was replaced with sterile PBS (the culture medium for *T. vitrinus*). Reaction mixtures were incubated overnight at 37°C. Addition of an equal vol of perchloric acid stopped the reaction and resulted in the precipitation of undigested protein which was pelleted by microcentrifugation at 11,000 g for 5 min using a microfuge. The absorbance of the resulting supernatant was measured at 405 nm using a microcentrifugal analyser (Instrumentation Laboratories, Warrington, Cheshire, UK). All reactions were carried out in triplicate.

2.9.9 Proteinase gelatin-substrate PAGE

Individual ES proteinases were visualised using a modification of the non-reducing discontinuous SDS-PAGE system where gelatin, a general substrate for proteinases, was incorporated into the polyacrylamide resolving gel mix before gel casting. After electrophoresis, the SDS was removed to enable re-association of the enzyme protein and restoration of enzyme activity. The gel was then incubated overnight in the appropriate buffer. During this incubation, the immobilised proteinases locally degraded the gelatin within the gel.

SDS-PAGE of ES samples was carried out as before except for the following modifications. Gelatin (0.1%) was incorporated into the resolving gels while samples for proteinase analysis were mixed with an equal vol of non-reducing SDS-PAGE loading buffer (section 2.4.2) and were not heated before loading into the wells of the gel. The SDS-PAGE buffer was chilled to 4°C before use and the gel tank was placed in a container and surrounded with ice during electrophoresis. Following electrophoresis, the gel was washed in 2.5% Triton X100 (3 washes, each for 15 min)

to elute the SDS. The gel was then incubated overnight in the appropriate buffer (as determined from the spectrophotomeric assay). Proteolytic activity was visualised as clear zones on a blue background after staining by Coomassie brilliant blue.

2.9.10 Inhibition studies

To investigate the effect of specific proteinase inhibitors on ES proteolytic activity the following inhibitors were used at the following final concentrations: the serine proteinase inhibitor, PmsF (1 mM); the thiol proteinase inhibitor, E64 (10 μM); the metallo-proteinase inhibitor, Na_2EDTA (10 mM); the aspartate proteinase inhibitor, pepstatin (1 μM). ES samples were incubated with the appropriate inhibitor for 1 hr on ice before they were loaded onto the gel for electrophoresis. The inhibitor was also included in the overnight incubation to ensure all the target proteinases were inhibited. Though PmsF has a short half-life (at pH 7.0, the half-time for hydrolysis is 100 min at 25°C), the inhibitor binds irreversibly to serine proteinases and inactivates the enzymes shortly after coming into contact with them (Price and Johnson, 1989).

2.9.11 Protein degradation by ES proteinases

To determine the type of proteins specifically degraded by the adult *T. vitrinus* ES proteinases, several proteins that would be expected to be found in the *in vivo* parasite environment, namely fibrinogen, plasminogen, albumin, haemoglobin, IgG, complement, myosin and fibronectin were tested as proteinase substrates.

Degradation reactions were carried out in a total vol of 70 μl, comprised of 10 μl crude protein preparation (10 mg/ml in deionised distilled water, stock, apart from complement which was 2.5 mg/ml stock), 5 μl concentrated adult *T. vitrinus* ES, 53 μl appropriate buffer. The antibiotics, penicillin and streptomycin, were added to a final concentration of 0.5 U/ml and 50 μg/ml respectively. For negative control reactions, the ES sample was replaced with sterile PBS. After overnight incubation at 37°C, the resultant preparations (20 μl) were analysed by 7.5% SDS-PAGE under reducing conditions (see section 2.9.2).

2.10 MICROBIOLOGICAL MANIPULATONS

Section A: General microbiological manipulations

2.10.1 Preparation and storage of bacterial stocks

For short term storage (<4 weeks) bacteria were streaked onto L-agar plates containing the required antibiotic. These were stored at 4°C.

For long term storage of bacterial stocks, bacteria were treated and stored using the following methods:

Glycerol stocks

A glass vial with a screw top lid (2 ml) containing 0.15 ml of glycerol was sterilised by autoclaving. An aliquot (0.85 ml) of the overnight culture was added to the sterile vial and the contents evenly mixed by gentle vortexing. The culture was rapidly frozen in an ethanol/dry ice mixture before being transferred to -70°C for long term storage. To recover the bacteria, a sterile inoculating loop was used to scrape the surface of the frozen culture and immediately streaked onto the appropriate agar plate. The plate then was incubated overnight at 37°C. The stock frozen culture was not allowed to thaw out and was returned to -70°C immediately.

Stab cultures

A glass vial (2 ml) was half-filled with molten LB-agar (section 2.4.4), the lid was fitted and the vial was sterilised by autoclaving. Once the vial had cooled and the agar had solidified a single colony from a bacterial plate was picked with a sterile needle and stabbed into the agar at the bottom of the vial. The lid was replaced and the culture was stored at room temperature in a light-tight box. The bacteria were recovered by stabbing a sterile inoculating loop into the vial agar and streaking onto an appropriate agar plate. The plate was incubated overnight at 37°C.

2.10.2 Growth of bacterial cultures

Overnight liquid cultures of *E. coli* bacteria were prepared by inoculating a single colony, picked from an LB-agar plate, into 10 ml of LB-broth (section 2.4.4) containing the appropriate antibiotic. The culture was grown overnight at 37°C in a gyratory incubator (200 rpm).

2.10.3 Preparation of competent cells

An overnight culture (containing no antibiotics) was prepared from a glycerol stock of *E. coli* strain JM 109. An aliquot of 1 ml of the overnight culture was used to inoculate 100 ml LB-broth (section 2.4.4) and this was incubated at 37°C with vigorous shaking, until the OD_{600} had reach 0.5 (approximately 2-3 hr). The culture was cooled on ice for 30 min before the cells were pelleted by centrifugation at 5,000 g for 10 min. The cells were resuspended in 50 ml chilled (4°C) 100 mM calcium chloride and stored on ice for 20 min. Finally, after pelleting the cells as before, they were resuspended in 3 ml of 100 mM calcium chloride. The cells were divided into 100 µl aliquots and those not being used immediately were added to 0.9 ml sterile glycerol and stored at -70°C.

2.10.4 Preparation of plating cells for lytic bacteriophage growth

A loopful of glycerol stock Y1090 was streaked onto a LB-agar/ampicillin plate (50 µg/ml) and incubated overnight at 37°C. A single colony was removed from the plate and inoculated into 10 ml of LB-broth (section 2.4.4) containing ampicillin (20 µg/µl) and was supplemented with sterile maltose solution (0.4% [w/v] final concentration). The culture was incubated overnight at 37°C with constant shaking. A 1 ml aliquot of the overnight culture was added to a 1 litre flask containing 50 ml of growth media (as before) and incubated at 37°C with vigorous shaking until the cells had grown to an OD_{600} of 0.5 (giving approximately 2.5×10^8 cells/ml). The culture was transferred to a 50 ml sterile tube and the cells were pelleted by centrifugation at 5,000 g for 10 min, as outlined above. The cells were resuspended in 15 ml of ice cold sterile 10 mM $MgSO_4$ and stored at 4°C for up to a week.

2.10.5 Preparation of plating cells for lysogenic bacteriophage growth

E. coli strain Y1089 was treated in the same way as the lytic plating cells, Y1090. Following growth of the 50 ml culture to an OD_{600} of 0.5, the culture was supplemented with 10 mM $MgCl_2$ and dispensed into 100 µl aliquots for use. Each 100 µl aliquot should contain approximately 2.2×10^7 cells.

Section B: Plasmid vectors and host cell manipulations

2.10.6 Subcloning of PCR-amplified DNA into the pCR 1000 and pCR II plasmid vectors

DNA fragments, amplified by PCR, may be directly subcloned into the Invitrogen pCR 1000 or pCR II plasmid vectors, as they utilise the single deoxyadenosines added to the 3'-end of PCR products by the enzyme *Taq* polymerase. All the components and the instructions required for ligation and subsequent transformation reactions were provided with the 'TA cloning system' kit (Invitrogen).

(i) ligation reaction

The kit instructions recommend that for ligations, the molar ratio of vector:PCR insert should be between 1:1 and 1:3. However, it was discovered that adding 'neat' PCR DNA, thus increasing the amount of insert to vector greatly, resulted in a substantial increase in ligation efficiency.

Sterile water (6 µl), 10x ligation buffer (1 µl), pCR 1000 or pCR II vector (2 µl; 25 ng/µl), PCR product (1 µl; undiluted) and T4 DNA ligase (1 µl) were mixed and the solution was incubated overnight at 12°C.

(ii) transformation reaction

β-mercaptoethanol (0.2 µl, 0.5 M) and ligation reaction (2 µl) were added to a vial containing 50 µl of competent *E. coli* cells (INFαF'). These components were mixed by gentle tapping. The vial was incubated on ice for 30 min followed by a heat shock incubation at 42°C for 60 sec, after which the cells were transferred directly

back to ice for 2 min. After the addition of 450 µl SOC medium (see section 2.4.4, pre-warmed to room temperature), the cells were incubated in a gyratory incubator (200 rpm) at 37°C for exactly 1 hr in order to allow expression of the selective marker. LB-agar/ampicillin (50 µg/ml) plates were prepared and 25 µl of X-gal (40 µl /ml stock, see section 2.4.1) was spread over them, after which the plates were allowed to stand for 1 hr to enable the X-gal to diffuse into the agar. The transformed cells were removed from the incubator and placed on ice. Two cell aliquots (25 and 100 µl) were spread onto separate X-gal LB-agar/ampicillin plates and incubated overnight at 37°C. Colonies carrying recombinant vector were selected by their white colour (inability to utilise the X-gal) compared to the blue colour of the hosts carrying non-recombinant vector.

2.10.7 Subcloning restricted DNA fragments into pBluescript II SK (+) plasmid vector

DNA inserts excised from recombinant λgt11 bacteriophage using *EcoR*1 were subcloned into the pBluescript II SK (+) plasmid vector from Stratagene which contains 21 unique restriction enzyme recognition sites (including *EcoR*1) within the *lacZ* gene. Ligation and transformation reactions were carried out according to the methods of Sambrook, Fritsch and Maniatis (1989).

(i) treatment of restricted pBluescript II SK (+) with calf intestinal alkaline phosphatase (CIP)

Prior to the ligation reaction the vector was linearised with *EcoR*1 to provide the correct ligation ends. To reduce the probability of the vector DNA recircularising during the ligation reaction, the *EcoR*1-restricted vector was treated with CIP, which removes terminal 5' phosphates.

CIP (0.1 unit) was added to the restricted vector (1 µl; 1 µg/µl) reaction and incubated at 37°C for 1 hr. After dephosphorylation, the vector was phenol extracted (to remove the CIP) and precipitated with ethanol. The vector was then resuspended in 20 µl of sterile water (final concentration approximately 50 ng/µl).

(ii) ligation

For ligation reactions a molar ratio of 2:1, DNA:vector was recommended in the manufacturer's instructions. CIP treated *Eco*RI ended pBluescript II (0.5 µl; 25 ng), 8 µl *Eco*RI cut DNA insert, 1.5 µl ligase buffer (0.5 M Tris-HCl buffer [pH 7.6], containing 100 mM $MgCl_2$, 100 mM dithiothreitol and 500 µg/ml BSA), and 4 µl sterile water were placed in a 0.5 ml sterile tube. This was mixed and incubated overnight at 12°C.

(iii) transformation

Competent JM 109 cells (100 µl) were added to 7.5 µl of the ligation reaction and the mixture was incubated on ice for 30 min. The mixture was transferred to 42°C for 2 min and then returned immediately to ice for 5 min. LB-broth (900 µl) was added to the cells and incubation was continued at 37°C in a gyratory shaker for 1 hr to allow expression of the selectable marker. LB-agar/ampicillin (50 mg/ml) plates were prepared by spreading individual plates with 20 µl each of 40 mg/ml X-gal and 40 mg/ml IPTG (see section 2.4.1) and these were allowed to diffuse into the agar for 1 hr at room temperature. Cells (100 and 200 µl) were spread onto LB-agar plates which were then incubated overnight at 37°C. The blue/white selection method allowed detection of the colonies containing recombinant vector.

Section C: Bacteriophage λgt11 and host cell manipulations

2.10.8 Creation of an adult *T. vitrinus* cDNA λgt 11 library

T. vitrinus cDNA λgt11 library was prepared from adult parasites (obtained from infected lambs, 21 days after infection) by Ms. D. Redmond, Moredun Research Institute, Edinburgh, UK in June 1990. The procedure followed was that of Amersham's 'cDNA cloning system - λgt11' kit. Adult *T. vitrinus* (obtained from infected lambs, 21 days after infection with *T. vitrinus* infective L3) cDNA was prepared according to the method described in the Nucleic Acid Manipulations section.

Briefly, the cDNA was treated with T4 DNA polymerase to ensure the cDNA was completely blunt ended. The cDNA blunt-ends were then converted to *Eco*RI cohesive ends by the ligation of *Eco*RI adapters onto the ends of the cDNA (see table 2.2). After the removal of unincorporated adaptors by a single column purification step, the cDNA *Eco*RI termini were phosphorylated with kinase. Following kinase treatment, an aliquot of the cDNA pool was analysed by agarose gel electrophoresis (see below) and was determined to contain cDNA fragments mainly in the size range 0.4 - 10 kb. The cDNA (approximately 100ng) was then ligated into *Eco*R1 restricted λgt11 bacteriophage (0.5 μg) and the ligation reaction was subsequently packaged *in vitro*. A sample of the recombinant phage was introduced into *E. coli* cells as described in the next section. The final adult *T. vitrinus* cDNA λgt11 library was contained in a volume of 0.5 ml and had a titre of approximately 2.5 x 10^5 pfu/0.1 ml (see below) with a cloning efficiency of 3.3 x 10^6 pfu/μg cDNA. The library was found to contain 25% recombinant phage.

2.10.9 Infection of Y1090 *E.coli* with bacteriophage λgt11

A 100 μl aliquot of Y1090 plating cells, as prepared in section A, was mixed with 100 μl of the appropriate dilution of bacteriophage (determined by phage titration as detailed below). Infection of bacteria with phage occurred by placing the mix at 37°C for 15 min. Molten top agar/agarose (5 ml, cooled to 43°C) was mixed with the bacteria/phage suspension and poured onto a LB-agar plate. After the top agar had solidified, the plate was inverted and incubated overnight at 37°C, or, for immunoscreening purposes (see later section), was incubated at 43°C until the phage plaques had just started to become visible (which took approximately 3-4 hr).

2.10.10 Titration of phage

To determine the correct phage dilutions required for the desired density of phage/plate, serial 10-fold dilutions of the phage stocks were prepared in SM buffer and, each dilution (100 μl) was used to infect Y1090 cells. The mixtures were plated as described in the previous section. All dilutions were carried out in triplicate. Phage titres were calculated by multiplying the total number of plaques present on the

plate by the dilution factor and were defined as the number of plaque forming units (pfu)/0.1 ml.

2.10.11 Preparation of antiserum for immunoscreening

Polyclonal antiserum may contain antibodies which bind to antigens produced by *E. coli*. Therefore, to avoid high background signals when immunoscreening the λgt11 cDNA library, the serum was preabsorbed with whole and lysed *E. coli*.

An overnight culture (10 ml) of Y1090 was lysed by the addition of 0.5 gm of lysozyme and the samples were incubated at room temperature for 10 min. The cells were freeze thawed three times and subsequently sonicated.

The required dilution of antiserum was prepared in 26 ml TBST, containing 5% horse serum. Whole and lysed cells (total 6 ml each) were added alternatively in 1 ml aliquots, gently shaking the solution after each addition for 20 min at room temperature. The whole and lysed cells along with the bound anti-*E. coli* antibodies were pelleted by centrifugation at 5,000 g for 20 min. The resulting supernatant was removed and used for immunoscreening.

2.10.12 Immunoscreening of λgt11 adult *T. vitrinus* cDNA library

Y1090 plating cells were infected with the adult *T. vitrinus* cDNA λgt11 library to give the required plating density as determined by titration of the library. The plates were incubated at 43°C until plaques were just visible (approximately 3.5 hr). Each plate was then overlaid with a dry nitrocellulose filter, previously saturated with 10 mM IPTG, which induced expression of recombinant peptides. Filter positions were marked with a needle before incubating the plates overnight at 37°C. All the following steps were carried out at room temperature with gentle agitation.

The filters were removed and washed four times (30 min each wash) with excess TBST (15 ml/filter), then blocked for 2 hr in 5% horse serum/TBS (5 ml/filter). After blocking, the filters were incubated overnight with *E. coli* antigen-absorbed, anti-adult *T. vitrinus* ES serum (as prepared above). The primary antibody was removed and the filters were washed five times (20 min each wash) with TBST before the addition of the second antibody, HRP conjugated to anti-rabbit IgG for 2

hr, at a dilution of 1:1000 in 5% (v/v) horse serum in TBST buffer. Following further extensive washing in TBST, a freshly prepared solution of DAB substrate (see section 2.4.1) was added. Once the colour had developed to the required intensity, the substrate was removed by washing in distilled water and the filters left to air dry. Positive plaques appeared dark brown in colour compared to the light brown background negative plaques. Dried filters were aligned with the original plates to enable identification of the positive plaques which were subsequently removed by coring with a sterile pipette tip and placed in 1 ml of SM buffer. 5 µl of chloroform was added to prevent bacterial growth and these phage stocks were stored at 4°C. Selected plaques were replated and screened again with the anti-ES antiserum until plaque purity was obtained.

2.10.13 Preparation of high titre λgt11 lysates

To provide an adequate amount of bacteriophage for further analysis phage lysates were prepared. The bacteriophage was resuspended in 100 µl of sterile water. A 1000-fold dilution of this phage was used to infect Y1090 and the mixture was plated, as described above. After the overnight incubation at 37°C almost confluent lysis of the bacterial lawn was achieved. The phage were eluted in 4 ml SM buffer (section 2.4.1) overnight at 4°C. The SM buffer, containing eluted phage, was removed and centrifuged at 5,000 g for 10 min to pellet bacterial debris. The supernatant (phage suspension) was removed into a new sterile tube, a drop of chloroform was added and the sample was stored at 4°C. The stock phage lysate contained approximately 1 x 10^9 pfu/0.1 ml.

2.10.14 Generation and isolation of recombinant lysogen

To obtain sufficient fusion peptide from recombinant λgt11 for visualisation in SDS-PAGE and analysis by Western blotting, lysogens in *E. coli* Y1089 were prepared. During the lysogenic growth phase, the phage DNA becomes incorporated and replicates as part of the host's genome. After the recombinant lysogen is grown to high density, IPTG is added to the medium resulting in the induction of *lacZ*-directed fusion protein.

A 100 µl aliquot of Y1089 plating cells was added to 100 µl of bacteriophage lysate and incubated at 32°C for 20 min. The infected cells (20 µl) were plated out on an LB-agar/ampicillin (50 mg/ml) plate and incubated at 32°C overnight. 16 colonies from the plate were selected and each was streaked onto two separated LB-agar/ampicillin plates. One plate was incubated overnight at 32°C and the other incubated at 43°C. Lysogens were identified by their ability to grow at 32°C but not 43°C.

Recombinant lysogens were inoculated into 10 ml LB-broth and incubated at 32°C with shaking until the optical density of the culture had reached 0.5 at 600 nm. At this point, 1.5 ml of the culture was removed to create a glycerol stock sample. The remaining 8.5 ml was transferred to a 43°C shaking incubator and incubated at the elevated temperature for 30 min. Two 1.5 ml aliquots were removed. The first was microcentrifuged at 11,000 g for 5 min and the pelleted cells were resuspended in 100 µl of SDS-PAGE Reducing Sample Buffer: this was the uninduced lysogen preparation. To the other 1.5 ml culture was added IPTG (to a final concentration of 10 mM) and the culture was incubated at 37°C for 60 min, after which the culture was centrifuged and the cells were resuspended in SDS-PAGE Reducing Sample Buffer. This was the induced lysogen sample. The lysogens were then analysed by SDS-PAGE as well as Western blotting.

2.10.15 Manipulation of *C. elegans* cholinesterase clones

The three *C. elegans* cholinesterase clones (cm7d7, cm10g6, cm06b1) originated from a *C. elegans* cDNA library (insert size 1-3 kb) constructed in the bacteriophage λSHLX2. They had been characterised by sequencing of a single strand from the 5' end of each clone, and 327, 465 and 503 nucleotides of cm7d7, cm10g6 and cm06b1 respectively, had been determined (Waterson *et al.*, 1992). The actual size of the clone recombinant DNA inserts were not known. The clones were provided as a single plaque suspended in 300 µl of Taqλ buffer (2.5 mM Tris-HCl buffer, pH 8.0, containing 2.5 mM $MgCl_2$ and 0.01% [w/v] gelatin). The DNA insertion site in λSHLX2 is flanked 5' by the SP6 promoter and 3' by the T7

promoter, therefore, to amplify the inserts by the PCR, primers were designed to the sense orientation of the SP6 promoter and the antisense orientation of the T7 promoter. PCR amplification of the λSHLX2 clones was carried out using the method of Saiki *et al.* (1986) as described in section 2.11.7.

2.11 NUCLEIC ACID MANIPULATIONS

2.11.1 Purificaton of nucleic acids by phenol:chloroform extraction

Proteins were removed from nucleic acid preparations by a phenol:chloroform extraction. An equal vol of phenol:chloroform (6:4) solution was added to the sample, which was then vortexed briefly and microcentrifuged for 10 min at 11,000 g. The upper aqueous phase (containing the nucleic acids) was removed and an equal vol of chloroform:isoamyl alcohol (24:1) solution was added. The mixture was then vortexed and centrifuged as before. This second step ensured that all the phenol was removed from the nucleic acid suspension. The upper aqueous phase was again removed and was transferred to a clean tube. The nucleic acids were precipitated with ethanol.

2.11.2 Ethanol precipitation of nucleic acids

DNA was precipitated from aqueous solutions either by (i) the addition of 1/10 vol 3M sodium acetate buffer, pH 5.2, and 2 vols ethanol, followed by storage of the mixture at -20°C for 1 hour, or, by (ii) the addition of an equal vol of 4 M ammonium acetate buffer, pH 6.4, and 4 vols of ethanol followed by storage on dry ice for 15 min. The DNA was pelleted by centrifugation at 10,000 g for 10 min, washed twice with 70% ethanol and dried under vacuum before dissolution in the required buffer.

2.11.3 Preparation of adult *T. vitrinus* genomic DNA

Adult *T. vitrinus* worms (0.5 gm wet weight, stored in liquid nitrogen) were powdered using a mortar and pestle (chilled to -70°C) and then solubilised in 5 ml of DNA Extraction Buffer (section 2.4.1). The solution was transferred to a 50 ml

sterile tube and incubated at 55°C for 15 min, followed by 1 hour at 37°C. DNase-free RNase (final concentration of 10 μg/ml) was added and the solution was incubated at 37°C for a further 15 min. An equal vol of phenol:chloroform (6:4) solution was added and the sample was then vortexed and microcentrifuged for 10 min at 11,000 g. The aqueous layer was removed and an equal vol of chloroform:isoamyl alcohol (49:1) solution was added, followed by vortexing and centrifugation as before. Again the aqueous layer was removed and the DNA was precipitated by adding 1/10 vol 3M sodium acetate buffer, 2 vols of 100% ethanol and the mixture was stored at -20°C for 2 hours. The DNA was pelleted by centrifugation (11,000 g for 20 min) and was dissolved in 1 ml of TE buffer.

2.11.4 Preparation of adult *T. vitrinus* RNA

Using a sterile mortar and pestle (chilled to -70°C), adult *T. vitrinus* worms (0.5 gm, wet weight) were homogenised in 2.5 ml of RNA Extraction Buffer (see 2.3.1). A further 2.5 ml of extraction buffer was added and the solution was left to warm to room temperature before being transferred to a sterile 50 ml tube. After the addition of sodium acetate buffer, pH 4.0 (final concentration 200 mM) and an equal vol of phenol:chloroform (6:4) solution, the mixture was vortexed, 1 ml of chloroform was added, the mixture was shaken vigorously for 5 minutes and then allowed to stand on ice for 10 min. Following microcentrifugation at 11,000 g for 20 min, the aqueous phase was removed, an equal vol of isopropanol was added and the RNA left to precipitate at -20°C for 1 hr. The RNA was pelleted by microcentrifugation at 11,000 g for 10 min, the supernatant was removed and the RNA was dissolved in 0.5 ml extraction buffer. An equal vol of isopropanol was added, the solution was mixed and then stored at -20°C for 1 hr. The RNA was pelleted as before, washed with 70% ethanol and was dissolved in 0.5 ml of sterile water.

Diethyl pyrocarbonate (DEPC), a strong inhibitor of RNAases, is commonly used to treat solutions and equipment used for RNA preparations (Sambrook, Fritsch and Maniatis, 1989). However, colleagues have previously found that DEPC-treatment of solutions and apparatus involved in the extraction and manipulation of

RNA from nematodes is not necessary (personal communication, Dr. D.P. Knox, Moredun Research Institute, Edinburgh, UK). Also, the RNA Extraction Buffer (see Solutions) contains 4 M guanidinium thiocyanate and β-mercaptoethanol, both of which inactivate RNAases (Sambrook, Fritsch and Maniatis, 1989).

2.11.5 Separation of polyadenylated RNA (messenger RNA) from non-polyadenylated RNA

Messenger RNA (mRNA) was purified from the total RNA preparation using the chromatographic technique of Aviv and Leder (1972). Essentially, total RNA was passed through an oligo-(dT) cellulose column. Non-polyadenylated RNA was removed by washing, and the purified mRNA was eluted with a low salt buffer.

Oligo(dT)-cellulose (0.3 gm) was equilibrated in sterile loading buffer (a solution of 20 mM Tris-HCl buffer, pH 7.6, containing 0.5 M NaCl and 1 mM Na_2EDTA) and poured into a sterile column. The final vol of column was 1 ml. The column was washed with 3-column vols each of sterile water, 0.1 M NaOH solution containing 0.5 M Na_2EDTA, and finally, sterile water, until the pH of the column effluent less than 8. Finally, the column was washed 5 vols of sterile loading buffer.

The total RNA solution was heated to 65°C for 5 min, an equal vol of 2x sterile loading buffer (40 mM Tris-HCl, pH 7.6, containing 1M NaCl and 2 mM Na_2EDTA) was added and the mixture was then allowed to cool to room temperature before being applied to the column (fraction 1). The flow-through was collected, reheated to 65°C, cooled to room temperature and reapplied to the column. Most of the non-polyadenylated RNAs and contaminants were removed in the resulting flow-through fraction. The remaining ribosomal RNA was eluted by further washing of the column with 4 vols of loading buffer (fractions 2-5). The transfer RNA was then eluted with 4 vols of low salt buffer (20 mM Tris-HCl, pH 7.6, containing 0.1 M NaCl and 1 mM EDTA) (fractions 6-9). Finally, polyadenylated mRNA was eluted by washing the column with 4 vols of sterile TE (fractions 10-17). The mRNA was precipitated by the addition of 1/10 vol 3 M sodium acetate buffer, pH 5.2, and 2 vols of ethanol followed by storage at -20°C for 1 hr. After pelleting the RNA by centrifugation, it was washed with 70% ethanol and resuspended in sterile water.

2.11.6 Synthesis of adult *T. vitrinus* complementary DNA

Complementary DNA (cDNA) was synthesised from purified mRNA using the cDNA Synthesis System Plus kit from Amersham. The method is based on that of Gubler and Hoffman (1983). The first strand cDNA copy was synthesised using reverse transcriptase and an oligo (dT) primer. *E. coli* RNase H was used to nick the RNA in the RNA-DNA hybrid. The second strand was generated using *E. coli* DNA polymerase, utilising the nicked RNA as a primer. The 3'-5' exonuclease activity of T4 DNA polymerase was used to remove any small remaining 3'-overhangs from the first strand cDNA.

(i) first-strand cDNA synthesis

All the components required, except the RNA, were provided in the kit. The following components were added to a sterile eppendorf in order given: 4 µl 5x concentrate of first strand synthesis reaction buffer, 1 µl sodium pyrophosphate solution, 1 µl human placental ribonuclease inhibitor, 2 µl deoxynucleoside triphosphate mix, 1 µl oligo (dT) primer and mRNA (2 µg resuspended in 9 µl sterile water). The contents were gently mixed, 40 units of reverse transcriptase (provided in kit and origin of the enzyme was not given) was added and this was followed by an incubation at 42°C for 40 min. After the incubation, the reaction was placed on ice. This completed the first strand reaction.

(ii) second strand cDNA synthesis

To the first strand reaction (20 µl) were added 37.5 µl second strand synthesis reaction buffer, 2 µl *E. coli* ribonuclease H (1.6 units), 13 µl *E.coli* DNA polymerase I and sterile water to make a final vol of 100 µl. The reaction was incubated at 12°C for 60 min, 22°C for 60 min and 70°C for 10 min. After a short centrifugation, the reaction was placed on ice and 4 units of T4 DNA polymerase added. This was gently mixed and incubated at 37°C for 10 min. The reaction was stopped by adding 4 µl of 0.25M Na_2EDTA, pH 8.0.

To quantify the cDNA, 1/10 of the cDNA preparation was analysed by DNA PAGE (section 2.11.10).

2.11.7 Polymerase chain reaction (PCR)

The polymerase chain reaction technique (PCR) mediates the amplification of a segment of DNA that is flanked by two regions of known sequence. Double-stranded DNA is first denatured to provide single-stranded DNA templates, the temperature is then lowered to allow annealing of two complementary oligonucleotide primers to the known flanking sequence, one to each strand, providing the starting point for synthesis of the new DNA strands and the reaction is then catalysed by the enzyme DNA polymerase. The cycle of denaturation, annealing and DNA extension is repeated several times resulting in the production of many copies of the central DNA segment.

The amplification procedure used was based on that of Saiki *et al.* (1986) which utilises the DNA polymerase from the thermophilic bacterium, *Thermus aquaticus* (Taq DNA polymerase). This enzyme can operate at high temperatures which allows the subsequent denaturation step to occur without inactivating the enzyme.

All PCRs were carried out in a reaction vol of 50 µl. Briefly, 5 µl of 10x PCR buffer (100 mM Tris-HCl buffer [pH 8.3], containing 500 mM KCl, 15 mM $MgCl_2$ and 0.1% [w/v] gelatin), 5 µl of each oligonucleotide primer (to give a final concentration of each as 0.2 mM) and 25 µl of DNA (10-50 ng) were mixed in a sterile 0.5 µl eppendorf. This mixture was placed in a boiling water bath for 5 min, then cooled on ice for 2 min before adding 10 µl of a stock solution of dNTPs (2 mM with respect to dATP, dCTP, dGTP and dTTP) and 1 unit of Taq DNA polymerase. The reactions were overlaid with 50 µl of mineral oil. The PCR reaction was performed using a Perkin Elmer Cetus DNA Thermal Cycler (Perkin Elmer Cetus, Norwallk, Conneticut, USA) over 30 cycles as follows: denaturation, 94°C for 1 min; primer annealing, 25-65°C (depending on the stringency required) for 2 min; primer extension, 72°C for 3 min. A further extension at 72°C for 8 min completed the amplification reaction.

The annealing temperature chosen depended on the melting temperature (T_m) of the primers. For each primer, the T_m was calculated by multiplying the number of A+T+I residues by 2°C and the number of G+C residues by 4°C and adding the two numbers together (Sambrook, Fritsch and Maniatis, 1989). For maximum stringency, the annealing was carried out at 5°C below the lower T_m of the two oligonucleotides used in the PCR reaction.

PCR amplification products (5 µl) were analysed either by agarose gel electrophoresis (section 2.11.8) or DNA-PAGE (section 2.11.10).

2.11.8 Agarose gel electrophoresis

Agarose gel electrophoresis was routinely used for analysis of DNA samples. Agarose is a linear polymer that, when solidified, forms an inert matrix for the DNA to pass through, the density of which is determined by the concentration of the agarose.

Nucleic acid grade "ultrapure" agarose, to give a final agarose concentration of 0.8% - 1.2% as desired, was dissolved in TAE buffer, and ethidium bromide was added to the agarose solution at a final concentration of 0.5 µg /ml before pouring the gel. DNA Sample Buffer (0.2 vol; see section 2.4.2) was added to the DNA samples before loading, and electrophoresis was performed using TAE buffer and a constant voltage of 1-4 V cm until the required resolution was achieved. 1 Kb DNA ladder (GIBCO-BRL, Life Technologies Ltd., Paisley, UK) was used as molecular weight standard. DNA fragments were visualised by the flourescence of bound ethidium bromide in UV light of 302 nm wavelength.

2.11.9 Recovery and purification of DNA from agarose gels

DNA fragments >200 bp were purified from agarose gels by the technique of Vogelstein and Gillespie (1979) using the commercially available "Gene Clean II" kit (Stratech Scientific Ltd., London, UK). Briefly, an agarose slice containing the required DNA fragment was excised from the gel, added to 3 vols of 3 M Sodium iodide and the mixture was incubated at 50°C until the agarose had melted. 'Glassmilk' suspension was added and the mixture was incubated at room

temperature (with occasional mixing) for 5 min to allow the DNA to bind to the glass. The DNA-glass milk complex was pelleted by centrifugation in a microfuge, the supernatant was removed and the pellet was washed three times with chilled (-20°C) "NEW wash". The DNA was then eluted into 20 µl of sterile distilled water by incubation at 50°C for 5 min.

2.11.10 Polyacrylamide gel electrophoresis for DNA

DNA was also analysed by continuous PAGE using the method of Herring *et al.* (1982).

Polyacrylamide gels (7.5%) were prepared by mixing appropriate vol of 30% acrylamide stock solution (section 2.4.1) with 5x concentration of Loening 'E' buffer (section 2.4.2) and distilled water. Ammonium persulphate and TEMED, were added immediately before the gel was poured, to final concentrations of 0.1% (w/v) and 0.1% (v/v) respectively. Before loading the DNA, Sample Buffer (0.2 vol; see section 2.4.2) was added to the samples. Gels were run in the 'mini protean' gel system (Biorad) using 1x Loening 'E' buffer at 200 V for 45 min.

2.11.11 Silver staining of polyacrylamide gels for DNA

DNA was visualised on the polyacrylamide gels by silver staining (Herring *et al.*, 1982). This is a much more sensitive technique for the visualisation of small (<500 bp) DNA fragments. After being fixed (an aqueous solution containing 10% [v/v] ethanol and 0.5% [v/v] acetic acid) for 10 min, the gel was incubated in silver nitrate (11.2 mM) for a further 15 min. The gel was quickly rinsed twice with distilled water and developed with a solution containing 750 mM NaOH and 0.1% (v/v) formaldehyde. Once the DNA bands had reached the desired intensity the reaction was stopped with 70 mM Na_2CO_3.

2.11.12 Electrophoresis of RNA through agarose gels containing formaldehyde

In order to denature and separate RNA for subsequent Northern blotting onto membrane the RNA was electrophoresed through a denaturing formaldehyde/agarose gel essentially as described by Fourney *et al.* (1988).

(i) Sample preparation

Adult *T. vitrinus* mRNA was prepared as described above. The mRNA (5 µl; 2 µg/5 µl) was added to sample loading buffer (15 µl). Sample buffer was prepared by mixing together the following volume of solution: 0.75 ml deionised formamide, 0.15 ml 10x MOPS buffer (section 2.4.2), 0.24 ml 37% formaldehyde, 0.1 ml sterile deionised, distilled water, 0.1 ml glycerol and 0.08 ml 10% (w/v in distilled water) bromophenol blue. The sample was heated to 65°C for 15 min then cooled to room temperature. Ethidium bromide (1 µl; 1 mg/ml) was added to the sample before it was loaded onto the gel.

(ii) Agarose gel preparation and electrophoresis

Ultra-pure agarose (1.25 gm) was dissolved in 10 ml 10x MOPS buffer and 87 ml sterile deionised distilled water. The agarose solution was allowed to cool to 50°C and 5.1 ml of 37% formaldehyde was added before the gel was cast. Electrophoresis was performed in 1x MOPS buffer at 100 V for 4 hours using Bethesda Research Laboratories (BRL; Life Technologies, Inc., Gaithersburg, MD, USA) model H5 gel electrophoresis system. High molecular weight RNA (0.24 - 9.5 kb; GIBCO-BRL) and low molecular weight RNA (0.16 - 1.77 kb; GIBCO-BRL) markers were co-electrophoresed with the mRNA sample.

2.11.13 Restriction enzyme digestion of DNA

Restriction enzyme digests were carried out in vol of 10-20 µl. All enzymes and their buffers were obtained from Boehringer Mannheim and the reactions carried out to the manufacturer's instructions. One unit of enzyme is usually defined as the amount required to digest 1 µg of DNA to completion in 1 hour using the recommended buffer and temperature (Sambrook, Fritsch and Maniatis, 1989). For restriction of DNA inserts from plasmid or bacteriophage digestions were carried out at 37°C for 2-3 hr, whereas, genomic DNA was digested overnight.

2.11.14 Small scale isolation of plasmid DNA

Small scale isolation of plasmid DNA from infected *E. coli* was performed by the alkaline lysis method described by Sambrook, Fritsch and Maniatis (1989), a modification of the method of Birnboim and Doly (1979). A single bacterial colony was inoculated into 10 ml LB-broth/ampicillin (20 μg/μl) and incubated overnight at 37°C with vigorous shaking. An aliquot of 1.5 ml was placed in a sterile eppendorf and centrifuged for 1 min to pellet the cells. The medium was removed by aspiration and the pellet was resuspended in 100 μl of 25 mM Tris-HCl buffer, pH 8.0, containing 50 mM glucose and 10 mM Na_2EDTA. After incubation at room temperature for 5 min, 200 μl of an aqueous solution containing 0.2 M NaOH and 1% SDS (w/v) was added and the solution was mixed by rapid inversion of the closed tube. The solution was stored on ice for 5 min before the addition of 150 μl of ice-cold, potassium acetate buffer, pH 4.8 (3 M potassium acetate [w/v] and 11.5% acetic acid [v/v] in distilled water). After vortexing, the solution was microcentrifuged for 5 min (11,000 g) and the resulting supernatant was transferred to a fresh tube. An equal vol of phenol: chlorofrom (6:4) was added, the solution was vortexed and the phases were separated by centrifugation at 11,000 g for 2 min. The aqueous phase was removed and 2 vol of ethanol added, mixed by vortexing and left to stand at room temperature for 5 min to precipitate the DNA. The DNA was pelleted by centrifugation, washed with 70% ethanol (v/v) and finally dissolved in 50 μl of TE (pH 8.0) containing DNase-free pancreatic RNase (20 μg/μl).

2.11.15 Di-deoxy chain termination sequencing of DNA

All sequencing reactions were carried out using the commercially available T7 sequencing kit (Pharmacia). Provided in the kit are all the components (except [^{35}S]-α-dATP) required to carry out DNA sequencing by the method of Sanger, Nicklen and Coulson (1977). Di-deoxy sequencing involves base-specific termination of primer-extension reactions which are catalysed by the enzyme T7 DNA polymerase. The method permits the primer extension reactions to be carried out in two stages, 'labelling' and 'termination'.

Preparation of double stranded plasmid template DNA was carried out by the alkaline lysis method, as detailed above: all subsequent steps were performed according to the manufacturer's instructions.

(i) annealing of primer to double-stranded template

Approximately 2 μg of plasmid DNA, contained in 32 μl of TE was denatured by adding 8 μl of 2 M NaOH and standing at room temperature for 10 min. The DNA was precipitated by the addition of 0.7 vol of water, 0.3 vol of 3 M sodium acetate buffer, pH 5.2 and 6 vols of ethanol, and the samples were stored at -20°C for 1 hr. After centrifugation, the precipitated DNA was washed with 70% (v/v in distilled water) ethanol, dried under vacuum and dissolved in 10 μl of sterile distilled water.

For the annealing step, 2 μl of annealing buffer (1 M Tris-HCl buffer, pH 7.6, containing 100 mM $MgCl_2$ and 160 mM dithiothreitol) and 2 μl of the sequencing primer (1.6 mM) were mixed with the DNA and incubated at 65°C for 5 minutes. The reaction was quickly transferred to a 37 °C water bath and was left for 10 min. The annealed template and primer were left at room temperature for at least 10 min before proceeding to the labelling and termination reactions.

(ii) sequencing reactions

An enzyme premix solution was prepared by mixing 1 μl of distilled water, 3 μl of "labelling mix A" (an aqueous solution containing 1.375 μM each of dCTP, dGTP and dTTP and 333.5 mM NaCl), 1 μl (37 kBq) of [^{35}S] dATP, and 2 μl of T7 polymerase (diluted to 1.5 units/μl in 'enzyme dilution' buffer, which is a solution of 20 mM Tris-HCl buffer, pH 7.5, containing 100 μg/ml BSA, 5 mM dithiothreitol and 5% [v/v] glycerol) for each template to be sequenced. To the annealed template and primer was added 6 μl of enzyme premix and the sample was incubated at room temperature for 5 min, during which time the radiolabelled dATP was incorporated into the newly synthesised DNA.

Chain termination was effected by adding 4 µl from this reaction to each of four tubes containing 2.5 µl of A, C, G and T mix respectively and incubating at 37°C for 5 min. Each mix consisted of the appropriate concentrations of dNTPs and ddNTPs contained in a solution of 40 mM Tris-HCl buffer, pH 7.6 and 50 mM NaCl. For example, the A mix consisted of 93.5 mM dATP, 840 mM each of dCTP, dGTP and dTTP, and 14 mM ddATP. Stop solution (5 µl of a 97.5% deionised formamide solution, containing 10 mM Na_2EDTA, 0.3% [w/v] each of xylene cyanol and bromophenol blue) was added to each tube prior to storage at -20°C.

(iii) preparation of sequencing gel and gel electrophoresis

A 6% acrylamide gel solution was prepared by mixing 210 gm urea, 72.5 ml 40% acrylamide stock solution (section 2.4.1), 50 ml 10 x TBE and bringing the volume to 500 ml with distilled water. For the actual sequencing gel, 0.8 ml of 10% (w/v, in distilled water) ammonium persulphate and 40 µl TEMED was added to 80 ml of the 6% acrylamide gel solution immediately before pouring the gel mix into the assembled glass plates. Electrophoresis was carried out using the S2 (BRL) or STS-45 (IBI) apparatus, at a constant power setting of 50 W or 70 W respectively. The gel was pre-run for 30 min before loading freshly denatured samples (heated to 80°C for 10 min). Further samples were loaded when the bromophenol blue in the previous samples had migrated off the end of the gel, and the gel was run for a maximum of 7 hr. After electrophoresis, the gel was fixed by soaking for 15 min in a solution containing 10% (v/v, in distilled water) methanol, 10% (v/v, in distilled water) acetic acid. The gel was then transferred to Whatman 3 MM filter paper and was dried on a gel drier under vacuum.

Detection of [^{35}S]-labelled nucleic acids in sequencing gels was carried out using X-ray film in autoradiography cassettes. Dried gels were placed in the cassette in direct contact with the film, and left overnight at room temperature. X-ray film was developed in X-ray developer, and fixed in fixer. The film was then washed in water before being air dried.

For recombinant inserts from selected immunopositive λgt11 clones, the PCR amplified fragments were sequenced directly (without subcloning) by Julia Bartley at Automated sequencing service, University of Durham, UK.

2.11.16 Southern blotting

DNA was transferred from agarose gels onto a nylon membrane by the capillary transfer technique developed by Southern (1975) and all the steps were carried out at room temperature. After electrophoresis the agarose gel was soaked for 45 min in several vols of an aqueous solution containing 1.5 M NaCl and 0.5 M NaOH with gentle agitation, to denature the DNA. The gel was then rinsed in distilled water before immersing in a neutralisation solution of 1 M Tris-HCl buffer (pH 7.4), containing 1.5 M NaCl, for 30 min. The neutralisation buffer was changed and the gel was soaked for a further 15 min. A piece of nylon membrane was cut to the same dimensions as the gel, moistened with distilled water and then was submerged in transfer buffer (10x concentration of SSC) for 10 min.

A solid support was covered with Whatman 3 MM paper and placed inside a large dish. The dish was filled with a 10x concentration of SSC until the level was just below the top of the support. Once the 3 MM paper was completely saturated with the buffer, the gel was removed from the neutralisation solution, placed in the centre of the support and surrounded with parafilm to prevent the transfer 'short-circuiting'. The nylon membrane was then placed on top of the gel. Both the gel and the membrane were marked to determine orientation. Two pieces of prewetted Whatman 3 MM paper were layered on top of the membrane followed by a stack of paper towels (approximately 10 cm high). Finally, a glass plate was placed on top of the towels and weighted down by a heavy object (approximately 1 kg). Transfer of the DNA was allowed to continue for 24 hr.

The apparatus was dismantled, the membrane was removed and was placed in a 6x concentration of SSC for 5 min to remove fragments of agarose sticking to it. To fix the DNA, the membrane was removed from the buffer, allowed to air dry and the side carrying the DNA was exposed to UV irradiation (254 nm) for 5 min using an ultraviolet transilluminator. The membrane was then ready for the hybridisation step.

The marker track of the Southern blot was removed before hybridisation for staining. After incubation in 5% (v/v, in distilled water) acetic acid for 10 min the marker strip was stained for 10 min with 0.04% (w/v) methylene blue in 0.5 M sodium acetate buffer, pH 5.2. The membrane was then destained in distilled water.

2.11.17 Northern blotting

Following electrophoresis of mRNA through formaldehyde agarose gels (see section 2.11.12), the mRNA was Northern blotted onto nylon membrane using the technique of Fourney *et al.* (1988). All steps were carried out at room temperature. On completion of electrophoresis, the agarose gel was immersed in several vols of 10x SSC for 20 min, with gentle shaking of the gel. The buffer was removed and the step was repeated. A piece of nylon membrane was cut to the dimensions of the gel was soaked in deionised distilled water for 5 min and then submerged in 10x SSC buffer for a further 5 min.

The transfer apparatus was assembled as described for Southern blotting. Transfer was carried out for 24 hr using 10x SSC as the transfer buffer. After transfer, the membrane was washed, the RNA was fixed and the marker tracks were stained, as detailed for Southern blotting.

2.11.18 Enhanced chemiluminescence random prime labelling of oligonucleotides and detection system

For Southern blot hybridisations of PCR reactions, the enhanced chemiluminescence (ECL) random prime labelling and detection system kit (Amersham) was applied. The labelling procedure is based on that of Feinberg and Vogelstein (1983) and introduces fluorescein-11-dUTP into the reaction which partially replaces dTTP. In subsequent stages, the fluorescein residues within the DNA are detected as haptens by anti-fluorescein HRP conjugate. Addition of 'detection reagent 1' produces hydrogen peroxide, the substrate for HRP. Reduction of this substrate is coupled to a light-producing reaction by 'detection reagent 2'. Oxidation of luminol present in the second reagent produces blue light which may be detected on a blue-light sensitive film. All the components required for the labelling

reaction and detection were provided in the kit and the protocol was as specified in the manufacture's instructions.

(a) labelling reaction

The oligonucleotide probe (50 ng in 20 µl) was denatured by heating for 5 min in a boiling waterbath and immediately transferring to ice for 2 min. After brief microcentrifugation, 10 µl 'nucleotide mix' (5 times concentration solution of Tris-HCl buffer, pH 7.8, containing fluorescein-11-dUTP, dATP, dCTP, dGTP and TTP $MgCl_2$ and β-mercaptoethanol, the concentrations of which were not given) , 5 µl 'primers' (random nonamers in an aqueous solution, the nucleotide sequence and concentration were not given) and 1 µl (4 units) of Klenow enzyme solution were added to the probe DNA. The solutions were gently mixed and incubated at 37°C for 4 hr. On completion of the reaction the level of incorporation was measured by removing 5 µl of the probe and spotting onto Hybond N^+ membrane. Unincorporated nucleotides were removed by washing the membrane in a 2 times concentration of SSC at 60°C for 15 min. The labelled probe was then visualised under UV light, and level of incorporation determined by comparison with various 5 µl dilutions of the nucleotide mix also spotted onto membrane (but not washed). Following the labelling reaction, the concentration of DNA was usually between 3-6 ng/µl.

(b) hybridisation

The Southern blot was placed in a Hybaid bottle (Hybaid, Teddington, UK.) and prehybridised at 0.25 ml/cm^2 in a 5 times concentrated solution of SSC containing 0.5% (w/v) blocking agent (supplied in kit, composition unknown), 0.1% (w/v) SDS, 5% (w/v) dextran sulphate and 100 µg/ml denatured sheared salmon sperm DNA, at 60°C for 1 hr in a Hybaid rotating oven. Unincorporated nucleotides were removed from the probe using a Nick Column (Amersham). The final labelled probe was denatured by heating in a boiling waterbath for 5 min before transferring to ice for 2 min. The blot hybridisation buffer was replaced and the denatured probe added. Hybridisation was allowed to continue at 60°C overnight in the rotating oven before

washing at room temperature with 2x SSC solution, containing 0.1% (w/v) SDS. The filters were then washed at 60°C with one of the following :1% SSC, 0.1% (w/v) SDS; 0.5x SSC containing 0.1% (w/v) SDS; 0.2x SSC, containing 0.1% (w/v) SDS. The lower the concentration of SSC in the wash buffer, the higher the stringency of the wash.

(c) blocking, antibody incubation and washes

The subsequent steps were carried out at room temperature with gentle agitation. Following the hybridisation washes, the filter was briefly rinsed (30 sec) in antibody wash buffer (100 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl) before blocking for 1 hr in 0.5% (w/v) 'blocking agent' (supplied with the kit, but the composition was not given) in wash buffer. The filter was rinsed again (for 30 sec) in the antibody wash buffer and then incubated for 1 hr with anti-fluorescein-HRP conjugate diluted 1000-fold in freshly prepared 0.5% (w/v) BSA in antibody wash buffer. Finally the blot was washed several times (4x 10 min washes) with the wash buffer.

(d) signal generation and detection

Equal vols of 'detection solution' 1 and 2 (supplied with the kit but the composition was not given) were combined (0.125 ml/cm^2 of filter), and the filter submerged in the mixture for exactly 1 min. The filter was then drained and covered with SaranWrap and placed in a film cassette in contact with an autoradiography film for up to 1 hr. The film then underwent development.

(e) further stringency washes

If required, after signal generation and detection, the filter was subjected to a higher stringency wash. Following detection, the filter was washed in 1 x SSC, containing 0.1% SDS at 60°C for 15 min. The filter was then washed at the desired stringency and the blocking, antibody incubation, washing and detection protocols were repeated (sections (c) and (d)).

2.11.19 PCR labelling of oligonucleotides with [^{32}P] dCTP, hybridisation and detection

For genomic DNA Southern and Northern blots, oligonucleotides were labelled with [^{32}P]-α-dCTP, providing a more sensitive probe than with the ECL system. The labelling procedure followed involved the use of a limited PCR amplification, as described by Hirst *et al.* (1992).

(a) labelling

The labelling reaction was carried out in a reaction vol of 50 µl and consisted of 100 pg/µl DNA template, PCR primer (1 µM each), 1x PCR buffer (a solution of 10 mM Tris-HCl buffer, pH 8.4, containing 50 mM KCl and 1.5 mM $MgCl_2$), dATP, dTTP, dGTP (50 µM each), unlabelled dCTP (100 pM) and 5 µl (1.85 Mbq) [^{32}P]α-dCTP. The mix was not boiled at any point and was overlaid with 50 µl of mineral oil. The reaction underwent 15 cycles of amplification as follows: 94°C for 1 min (denaturation); 55°C for 1 min (primer annealing); 72°C for 1 min (primer extension) and a final primer extension at 72°C for 7 min.

(b) hybridisation and detection

Prehybridisation, purification and denaturation of probe, hybridisation and subsequent washes were carried out exactly as described for the ECL system, apart from the fact that the composition of the hybridisation buffer which was as follows: 7% SDS (w/v) in 0.5 M $NaHPO_4$ buffer, pH 7.2 (adapted from Church and Gilbert, 1984).

After the final wash, the filter was wrapped in SaranWrap and placed in a film cassette in contact with autoradiography film for a period up to 10 days.

2.11.20 Computer analysis of DNA sequences

DNA sequences and their subsequent peptide sequences were analysed using the Daresbury 'SEQNET' computer databases (Daresbury Laboratory, Daresbury, Warrington, UK) and the University of Wisconsin Genetics Computer Group programs (Devereux *et al.*, 1984; GCG, 1991). The best alignments to DNA sequences were sought using the 'FastA' programme (aligorithm of Wilbur and Lipman, 1983; Pearson and Lipman, 1988) which scans both EMBL and Genbank databases. Translation of the DNA sequences were carried out using the 'MAP' command (Schroeder and Blattner, 1982) and these deduced peptide sequences were analysed using the 'FastA' (aligorithm of Wilbur and Lipman, 1983; Pearson and Lipman, 1988) or 'DAP' search of the SwissProtein database. Multiple peptide and nucleotide sequences were aligned using 'PILEUP', a simplification of the progressive alignment of Feng and Doolittle (1987).

Chapter three

Partial biochemical characterisation of acetylcholinesterase and proteinases excreted/secreted *in vitro* by *T. vitrinus*

3.1. INTRODUCTION

The definition of parasite enzymes excreted/secreted *in vitro* has contributed greatly to the understanding of how parasites manipulate their host environment and has identified possible target proteins for serodiagnosis, anthelmintic control and vaccination. Nematode secretory enzymes identified include AChE (e.g. Ogilvie *et al.*, 1973; Blackburn and Selkirk, 1992), proteinases (e.g. Knox and Kennedy, 1988; Britton *et al.*, 1992) and superoxide dismutase (SOD, eg. Knox and Jones, 1992; Britton *et al.*, 1994) and have been assigned many essential roles in parasite maintenance within the host. Previously, the parasitic stages of *T. vitrinus* maintained *in vitro* have been shown to release AChE, proteinases and SOD (Knox and Jones, 1990, 1992), although the biochemical and molecular properties of these enzymes were largely undefined. This chapter focuses on the further characterisation of AChE and proteinase enzymes secreted *in vitro* by *T. vitrinus*.

3.1.1 Acetylcholinesterase

AChE (EC, 3.1.1.7) is a serine esterase that hydrolyses acetylcholine (ACh) into choline and acetate. The enzyme is an essential catalyst in the regulation of muscular movement within nematodes, rapidly degrading ACh after transmission at neuromuscular junctions and restoring the post-muscle end plate membrane to its former polarised state. Neural AChE is the target of many carbamate and organophosphate nematicides causing hyperactivity, discoordination and eventually paralysis of the parasite (Chang and Opperman, 1991).

AChE has also been found to be actively secreted by several nematodes, cultured *in vitro*: *N. brasiliensis* (Lee, 1970; Jones and Ogilvie, 1972), *T. colubriformis* and *T. axei* (Ogilvie *et al.*, 1973), *Oesphagostomun radiatum and Oesphagostomum venulosum* (Bremner *et al.*, 1973), *Stephanurus dentatus* (Rhoads, 1981), *B. malayi* (Rathaur *et al.*, 1987), *T. vitrinus* (Jones and Knox, 1990), *D. viviparus* (McKeand *et al.*, 1994 a and b).

Evidence for the secretion *in vivo* of AChE in *Trichostrongylus* spp. was demonstrated by Rothwell, Ogilvie and Love (1973) and Jones and Knox (1990).

Antibodies from sheep infected with *T. colubriformis* bound to worm AChE (Rothwell, Ogilvie and Love, 1973), though enzymatic activity was not affected (Rothwell and Merritt, 1974). Two AChE isoenzymes present in intestinal mucosa homogenates from sheep infected with *T. colubriformis* were similar (based on electrophoretic mobilities) to isoenzymes from adult worm homogenates and were not detected in uninfected control animals (Jones and Knox, 1990).

The role of secretory AChE (sAChE) in the maintenance of the parasite within the host is unknown, but several functions have been proposed (reviewed by Philipp, 1983; Rhoads, 1984; Pritchard, 1993 a). Intestinal motility and secretion are both under parasympathetic (cholinergic) control. It has been proposed that sAChE may act as a 'biochemical holdfast' locally inhibiting peristalsis by ACh breakdown, helping the worms to resist expulsion (Lee, 1970), although this would not be such an essential requirement in AChE-secreting nematodes that physically attach to the intestinal wall, for example, *N. americanus* (Pritchard, 1993 a). Furthermore, Foster, Dean and Lee (1993) have more recently suggested that inhibition of peristalsis may be due to a porcine valosin peptide analogue that has been found in the ES of *T. colubriformis* (Savin *et al*, 1990). Degradation of ACh would also reduce the secretion of mucus from goblet cells, thereby reducing the efficiency of mucus to trap worms (Philipp, 1983).

ACh and other related agonists enhance immunological triggering of histamine release by mast cells and basophils (Kaliner and Austen, 1975; Hirata, Axelrod and Crews, 1979), lysosomal enzyme secretions (Ignarro and Colombo, 1973), neutrophil chemotaxis (Hill *et al.*, 1975) and antibody-dependent cellular cytotoxicity (Gale and Zighelboim, 1974). Parasitic sAChE could therefore play an important role in disabling the hosts cellular effector response.

sAChE may provide precursors for nematode metabolism, particularly choline, as acetate is a by-product of respiration. Choline is utilised in phosphatidylcholine, an important membrane lipid. Pritchard (1993 a) noted that in *A. caninum*, which does not secrete AChE, the predominant lipid is phosphatidyl ethanolamine and not phosphatidylcholine as seen in other nematodes, and suggests that an AChE deficiency in some nematodes may render them unable to utilise choline.

AChE has also been shown to have a potential application as a serodiagnostic marker of intestinal trichostrongylosis (Jones and Knox, 1990), because the enzyme is not secreted in such quantity by other co-infecting GI nematodes (Ogilvie *et al.*, 1973; Knox and Jones, 1990). Watts *et al.* (1982) noted that BZ reduced AChE secretion in *N. brasiliensis*. Following this, AChE secretion *in vitro* has been proposed as the basis of a possible assay for anthelmintic screening (Rapson, Chilwan and Jenkins, 1986). Sutherland and Lee (1993) observed that BZ-resistant *H. contortus*, *O. circumcincta* and *T. colubriformis* secreted higher levels of AChE than BZ-susceptible worms. BZ is known to affect parasite tubulin (Lacey and Pritchard, 1986) but it is unclear why AChE would be linked to BZ resistance. Perhaps AChE interferes with the binding of BZ to tubulin or alters the BZ in the nematode (Sutherland & Lee, 1993).

If secretion of AChE is one level of immune modulation by the parasite, it is feasible that it is an important target of the host immune response. The usefulness of AChE, purified from *T. colubriformis* as a protective antigen was assessed by Rothwell and Merritt (1975) in guinea-pigs (the laboratory model for the infection) but significant protection was not observed. In view of the presumed roles the enzyme plays in the survival of *Trichostrongylus* spp. within the host the result was somewhat surprising. However, this may be explained by the fact that a single dose of enzymatic protein without adjuvant was administered subcutaneously prior to challenge with the parasite and perhaps the enzyme was not sufficiently immunogenic. A protective response may have been observed if the enzyme had been administered with adjuvant or alternatively, directly to the GI mucosa over a prolonged period, more in keeping with the conditions of exposure of the host to the antigen which prevail in the natural course of infection. More recently, Griffiths and Pritchard (1994 b) performed a protection trial in which purified AChE from *T. colubriformis* was used as a candidate vaccine against mixed *T. colubriformis*, *H. contortus* and *Cooperia onocophora* challenge infections of sheep. No significant protection was observed in a challenge infection with *T. colubriformis* alone but some protection was found in a mixed worm challenge infection. The authors suggest that a change in adjuvant and improving the delivery system may enhance the immunogenicity of

sAChE. In addition, McKeand *et al.* (1994 b) showed that guinea-pigs vaccinated with *D. viviparus* ES enriched for AChE were significantly protected against *D. viviparus* challenge infection compared to guinea-pigs vaccinated with adjuvant only.

3.1.2 Proteinases

Proteolytic enzymes (proteinases) catalyse the hydrolysis of peptide bonds within protein molecules. Secretion of proteolytic enzymes by parasitic helminths during *in vitro* culture is well documented and is often stage-specific (e.g. Lackey *et al.*, 1989; Lawrence and Pritchard, 1993; Young, Knox and McKeand, 1995). Proteinases may be classified into 4 main groups, distinguished by different functional amino acid residues and their configuration within the active site (Neurath, 1989). All 4 types have been identified in the ES products of parasitic nematodes:

(i) **Serine proteinases** such as those secreted *in vitro* by L3 *Onchocerca lienalis* and microfilariae of *Onchocerca cervicalis* (Lackey *et al.*, 1989), adult *Nematospiroides dubius* (Monroy *et al.*, 1989) and L4 *O. circumcincta* (Young, Knox and McKeand, 1995).

(ii) **Cysteine (thiol) proteinases** are predominately secreted by *H. contortus* (Karanu *et al.*, 1993) and adult *Strongylus vulgaris* (Caffery and Ryan, 1994).

(iii) **Metallo-proteinases** were found in the ES of microfilariae *O. cervicalis* (Lackey *et al.*, 1989), L3 and adult *N. brasiliensis* (Healer, Ashall and Maizles, 1991), adult *D. viviparus* (Britton *et al.*, 1992) and adult *O. circumcincta* (Young, Knox and McKeand, 1995).

(iv) **Aspartate (carboxyl) proteinases**. This group has been identified in ES components of larval and adult *A. suum* (Knox and Kennedy, 1988), adult *D. viviparus* (Britton *et al.*, 1992) and adult *O. circumcincta* (Young, Knox and McKeand, 1995).

Host antibody inhibition of ES proteinases from *A. suum* (Knox and Kennedy, 1988) and *D. viviparus* (Britton *et al.*, 1992) provides evidence for the secretion of the proteinases *in vivo* and also demonstrates the potential of these enzymes to elicit a protective response within the host.

Secreted proteinases potentially play an essential part in the parasite's survival *in vivo* and are frequently stage-specific reflecting the changing environment encountered within the host during parasite development. The infective L3 stage of *H. polygyrus* releases a proteinase that may be required for ecdysis after ingestion of the parasite by the host (Lawrence and Pritchard, 1993). Gamble, Purcell and Maizles (1989) isolated a zinc metallo-proteinase from the L3 stage of *H. contortus* that may be necessary for L3 exsheathment. The secretion of proteinases to aid the penetration of the host tissue barriers (Knox and Kennedy, 1988; McKerrow, 1989; Healer, Ashall and Maizels, 1991; Britton *et al.*, 1992) is exemplified by the collagenolytic and elastolytic activities released *in vitro* by the L2 and L3/4 stages of *A. suum* (Knox and Kennedy, 1988). McKerrow (1989) noted that tissue-penetrating parasite stages secrete mostly metallo- and serine proteinases.

Breakdown of host tissue could provide the parasite with nutrients, though proteinases may also be secreted specifically for the purposes of gaining nutrients. Blood-feeding nematodes such as *H. contortus* (Karanu *et al.*, 1993) and *S. vulgaris* (Caffery and Ryan, 1994) release cysteine haemoglobinases into their environment and the hookworm, *Ancylostoma caninum* secretes an anticoagulant (Hotez and Cerami, 1983). A potential secretory anticoagulant (fibrinogen degradation) proteinase was also detected in *O. circumcincta* (Young, Knox and McKeand, 1995), a nematode which is not an obligate blood-feeder. Evasion of the host defence mechanisms by parasite proteolysis of molecules involved in immunity has also been suggested. ES proteinases from *S. mansoni* (Auriault *et al.*, 1981) and the trematode, *Fasciola hepatica* (Chapman and Mitchell, 1982), have been shown to cleave IgG suggesting that the enzymes may actively remove immunoglobulin molecules bound to the surface of the parasite. Also, *S. mansoni* serine proteinases have been shown to regulate the synthesis of host IgE (Verwaerde *et al.*, 1988). The inactivation of complement components and leukocyte cytotoxic mediators by parasite proteinases may also serve in attacking host immune responses (Leid, Suquet and Yanigoshi, 1987).

The secretion of AChE (Jones and Knox, 1990) and proteinases (Knox and Jones, 1990) by *T. vitrinus* has been recorded. In this chapter, the partial biochemical

characterisation of AChE and proteinases released *in vitro* by adult *T. vitrinus* is described. The enzymes were defined on the basis of their pH optima, molecular size, substrate specificity and sensitivity to specific inhibitors. Also, an initial characterisation of the proteinases secreted by the L4 stage of *T. vitrinus* was implemented.

3.2 RESULTS

3.2.1 AChE analysis of adult *T. vitrinus* ES

(a) pH optimum and eserine inhibition

Total AChE activity present in the ES of adult *T. vitrinus* was measured spectrophotometrically at 37°C by the method of Ellman *et al.* (1961, see section 2.9.5) utilising ATCI as the substrate. Enzyme activity was measured over a range of pH buffers (pH 5.0 - 9.0) in order to establish the optimal pH conditions for the secreted AChE. The resultant pH profile is shown in figure 3.1. AChE was active over the a broad pH range with a distinct peak of activity at pH 7.5.

The spectrophotomeric assay was repeated at optimal pH (pH 7.5) in the presence of eserine (100 mM - 0.1 mM), a specific inhibitor of AChE, to determine that the activity seen was due to AChE alone and not pseudocholinesterases. The results of the eserine inhibition study are shown in table 3.1. Cholinesterase activity was completely inhibited at all the concentrations of eserine tested, confirming that the esterase activity present in the adult ES is due solely to AChE.

(b) Visualisation of esterase and AChE profiles by native PAGE

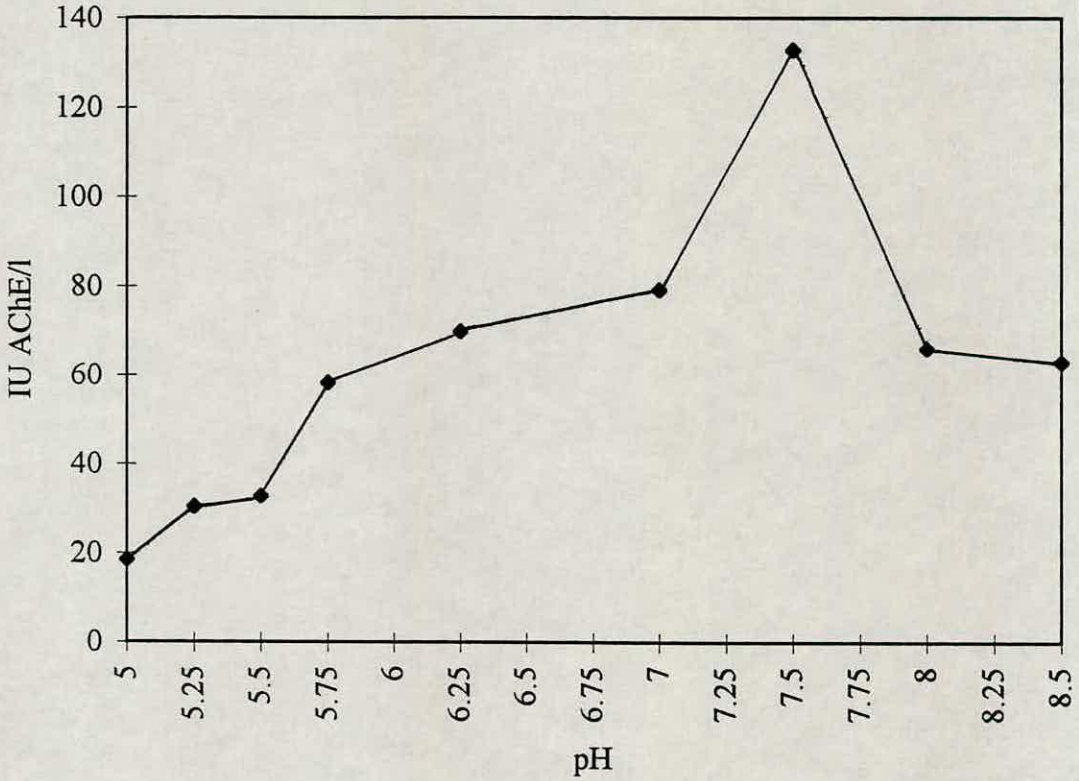
Having established the optimum pH for assaying AChE, activity was examined zymographically, following fractionation of concentrated adult *T. vitrinus* ES by native PAGE (section 2.9.2), gels were stained for protein, esterase (Grunder, Sartori and Stormont, 1965; section 2.9.6) and AChE (Karnovsky and Roots, 1964; section 2.9.7) (figure 3.2). Adult *T. vitrinus* homogenate samples were included as positive controls to establish that the ES had been concentrated sufficiently to enable the visualisation of protein and enzyme activities by this method. The samples that were stained for AChE and esterase activities were run on the same gel so that a direct comparison could be made.

The homogenate protein appeared as a streak across the gel (protein, lane H), while the ES proteins were concentrated in the upper region of the track (protein, lane ES). Esterase activity in the homogenate (esterase, lane H)) was evident as 2 main

bands, the upper band being AChE (AChE, lane H). In addition, a rapidly migrating zone of activity was evident. A similar band of AChE activity was seen in the concentrated ES material (AChE, lane ES) and corresponded to the region where most of the protein was evident. Esterase activity in the homogenate (Esterase, lane H) was evident as two sharply defined bands at the top of the gel and a rapidly migrating diffuse zone two thirds of the way down. Only the upper sharply defined band was visualised by AChE staining (AChE, lane H). By contrast, a sharply defined band at the top of the gel and a moderately migrating zone of enzyme activity visualised by esterase staining of ES (Esterase, lane ES) were both visualised by AChE staining (AChE, lane ES), although the more rapidly migrating activity did not have coincident electrophoretic mobility with the zone observed after esterase staining (Esterase, lane ES). The AChE did not migrate very far into the gel suggesting that it is of high molecular weight or exists in a complex. Nematode sAChEs tend to be either monomeric or exist as multiple molecular forms, (Chang and Oppermann, 1991). The molecular weight of the proteins could not be clearly established under native conditions.

Figure 3.1

pH profile of adult *T. vitrinus* ES AChE activity



Total AChE activity of adult *T. vitrinus* ES (unconcentrated, approximately 0.1 mg protein/ml) was monitored using a range of buffers over the pH range 5.0 - 8.5. AChE activity was expressed as IU AChE/l.

Table 3.1

Eserine inhibition of adult *T. vitrinus* sAChE.

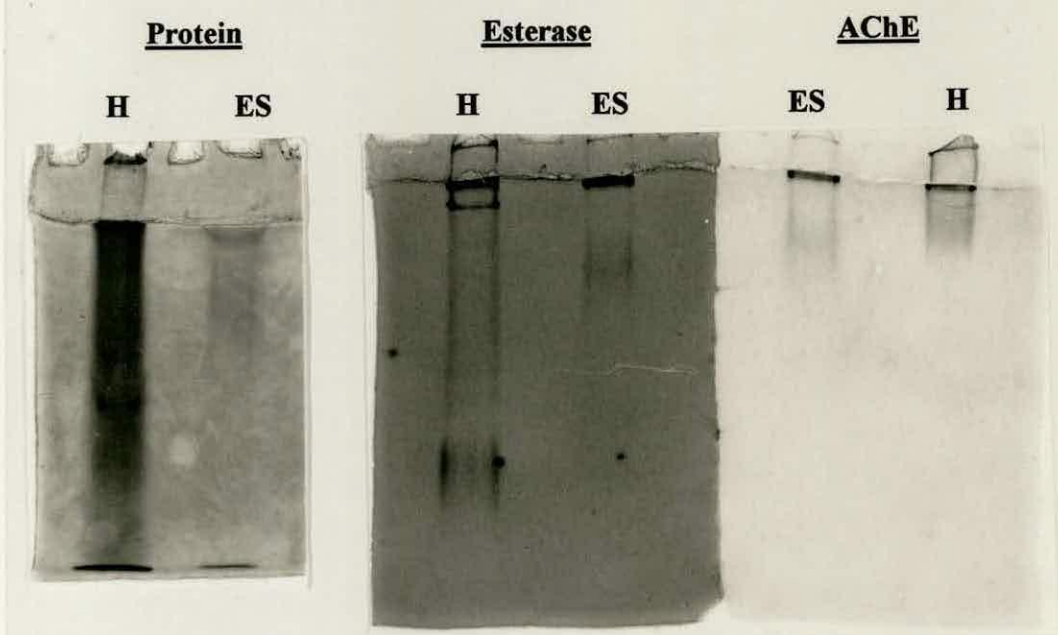
concentration of eserine (mM)	IU AChE/l
100	0
10	0
1	0
0.1	0
0	120

Activity of AChE in adult *T. vitrinus* ES (unconcentrated, approximately 0.1 mg protein/ml) was measured using the method of Ellman *et al.* (1961) at optimal pH (pH 7.5) in the presence of the AChE specific inhibitor, eserine (100 - 0.1 mM).

Figure 3.2

Protein, esterase and AChE native PAGE profiles of adult *T. vitrinus* homogenate and ES.

Adult *T. vitrinus* homogenate (H, 50 μg protein/track) and ES (2-10 μg protein/track) were fractionated by 7.5% native PAGE (section 2.9.2). Tracks of H and ES were either stained for protein, esterase (Grunder, Sartori and Stormont, 1965) or AChE (Karnovsky and Roots, 1964). Under native PAGE conditions the proteins do not migrate according to molecular size.


Protein
H
ES
Esterase
H
ES
AChE
ES
H

3.2.2 Proteinase analysis of adult *T. vitrinus* ES

(a) pH optimum

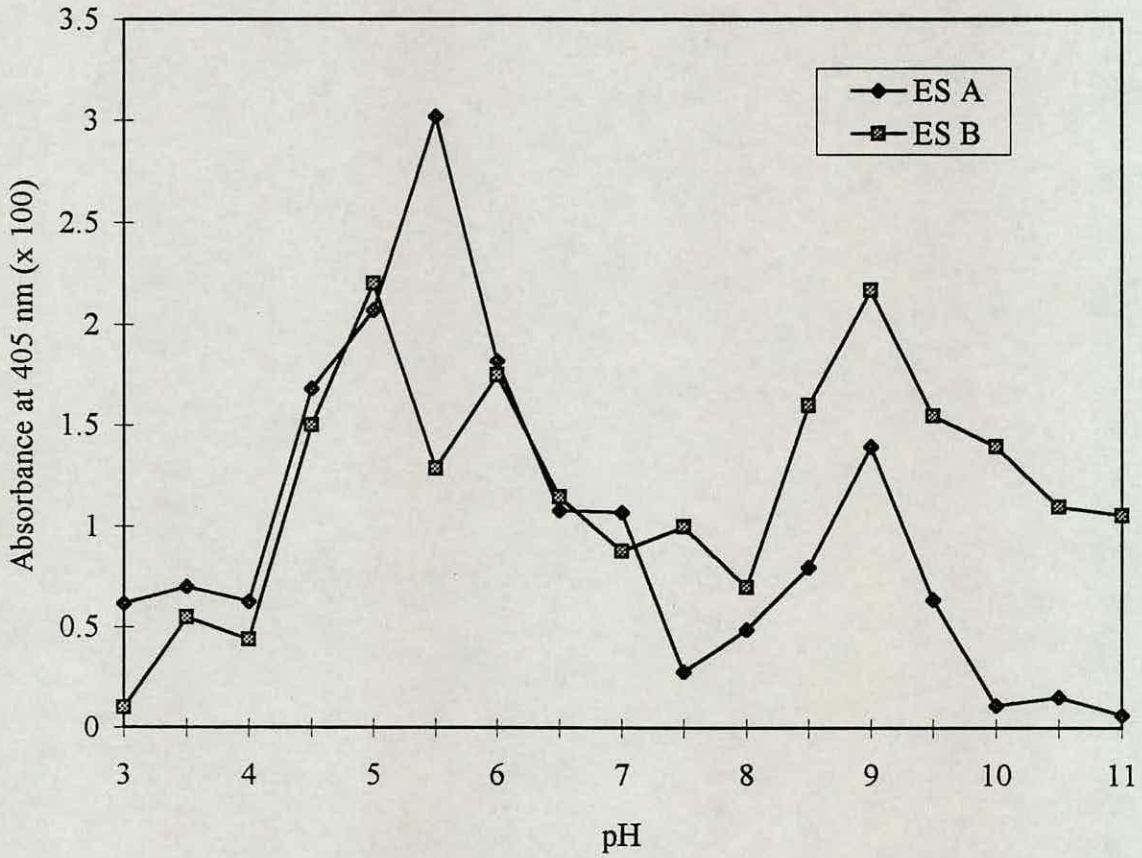
The effect of pH (3.0 - 10.5) on total proteolytic activity of ES adult *T. vitrinus* ES was determined essentially as described by Knox and Kennedy (1988) using azocaesin as substrate (section 2.9.8). The pH profile was assessed on two occasions with different batches of ES and is shown in figure 3.3.

In general terms, both batches of ES showed optimal activity at acidic (pH 4.5 to 6.5) and alkaline (pH 8.5 to 9.5) pH values.

Differences were observed in the pH profile between batches of ES. Most notably, a distinct biphasic peak was evident with ES B at acidic pH although a shoulder was observed on the main peak of activity (pH 5.5) in ES A between pH 4.5 and 5.0. Both ES batches had the same alkaline optimum, pH 9.0.

Figure 3.3

pH profile of adult *T. vitrinus* ES proteinase activity.



The total proteolytic activity of adult *T. vitrinus* ES (0.3 mg protein/ml) was assesses over a range of pH (pH 3.0 - 11.0) by the method of Knox and Kennedy (1988, see section 2.9.8) utilising azocaesin as substrate.

(b) Gelatin-substrate gel analysis and inhibitor sensitivities

The migration of individual proteinases was estimated following fractionation of ES through 7.5% polyacrylamide gels, containing gelatin, under non-reducing SDS-PAGE conditions (see section 2.9.9). The results can be seen together with a corresponding protein profile, stained with Coomassie blue stain (section 2.9.3) for comparison (figure 3.4). Separation of ES proteins by non-reducing SDS-PAGE (figure 3.4) gave a profile of 18 to 20 proteins with the most prominent bands at approximately 210, 200, 50 and 36 kDa.

Incubation of specific proteinase inhibitors with the ES samples before gel electrophoresis and in the overnight gel incubation buffers allowed the identification of classes of proteinases present. Proteolysis was monitored under acidic (figure 3.5 (a)), neutral (figure 3.5 (b)) and alkaline (figure 3.5 (c)) conditions. Though the optimal pH for ES proteinase activity was found to be pH 5.0 - 5.5, as determined by the above spectrophotomeric assay (see figure 3.3), proteinases which were active at neutral and alkaline pH were also observed by gelatin-substrate analysis (figures 3.5 a-c).

At pH 5.5 (figure 3.5 (a)), principal zones of proteolysis were observed at 102, 59 and 53 kDa (lane C) with fainter bands evident at approximately 205 and 104 kDa and a smear at 66 kDa. The pattern was repeated at pH 9.0 (figure 3.5 (c), lane C). At pH 7.0 proteolytic activity was visualised at 53 and 102 kDa (figure 3.5 (b), lane C) although fainter bands of proteolysis were also observed on the actual gel at 104 and 59 kDa.

In the presence of the serine proteinase inhibitor, PmsF, activity at 102 kDa region was markedly inhibited at all pHs (figures 3.5 a-c, lane PmsF) suggesting the presence of a serine proteinase. Incubation of the proteinases in the presence of the metallo-proteinase inhibitor, EDTA, completely abolished the activity of the 59/53 kDa doublet at pH 5.5 and pH 7.5 (figures 3.5 (a) and (b), lane EDTA) but not at pH 9.0 (figure 3.5 (c)). The fainter proteinases at 205, 104 and 66 kDa were relatively unaffected by the proteinase inhibitors chosen. The cysteine proteinase inhibitor, E64, and the aspartyl-proteinase inhibitor, pepstatin, did not inhibit any of the proteinases observed (figures 3.5 (b) and (c), lanes E64 and Peps). However, at pH 7.5 these

inhibitors appeared to enhance the activities of the 59/53 kDa metallo-proteinases. Moreover, additional activities at approximately 28 kDa and 45 kDa were evident in the presence of E64 and pepstatin at pH 7.5 (figure 3.5 (b), lanes E64 and Peps).

Figure 3.4

Protein non-reducing SDS-PAGE profile of adult *T. vitrinus* ES.

Adult *T. vitrinus* ES (2-10 μg protein/track) was fractionated using 7.5% SDS-PAGE and non-reducing conditions (section 2.9.2) followed by Coomassie blue staining.

Lanes:

ES - adult *T. vitrinus* ES protein profile
M - high molecular weight protein markers

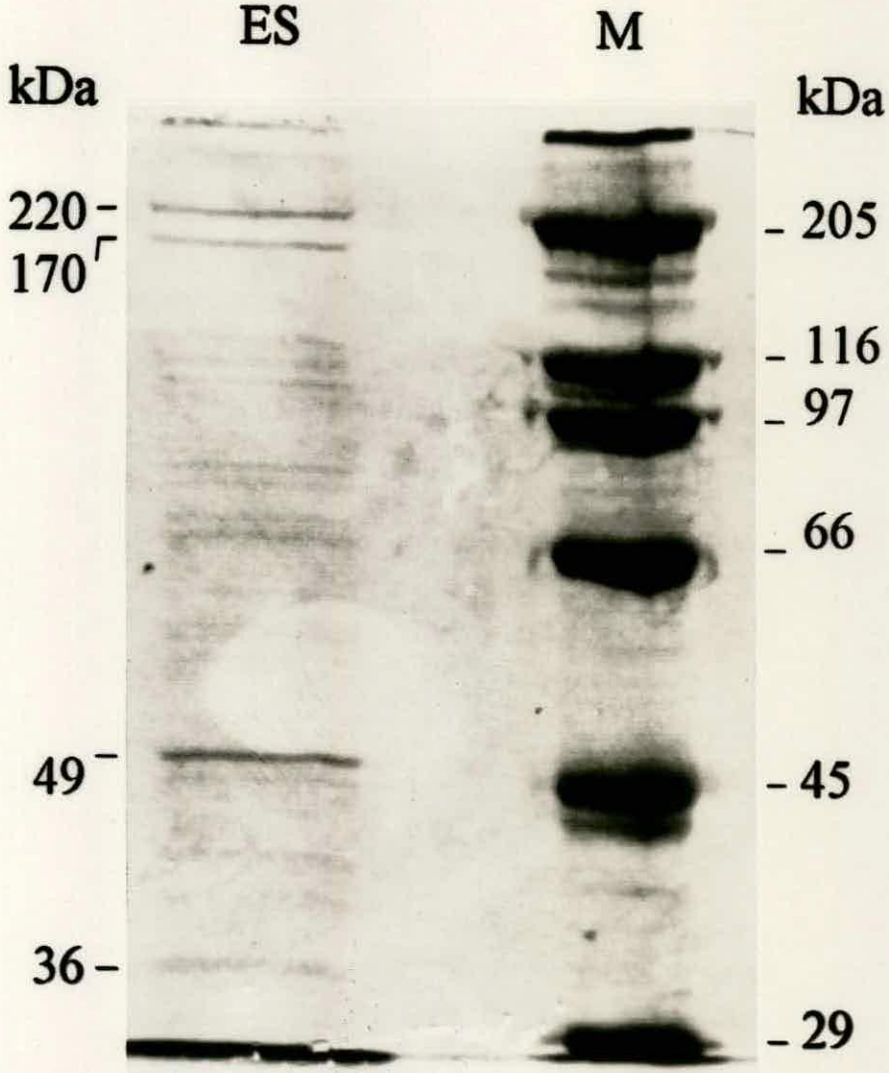

ES
M
kDa
kDa
220
170
49
36
205
116
97
66
45
29

Figure 3.5

Gelatin-substrate gel analysis and inhibitor sensitivities of the ES proteinases of adult *T. vitrinus* at (a) pH 5.5, (b) pH 7.5, and (c) pH 9.0.

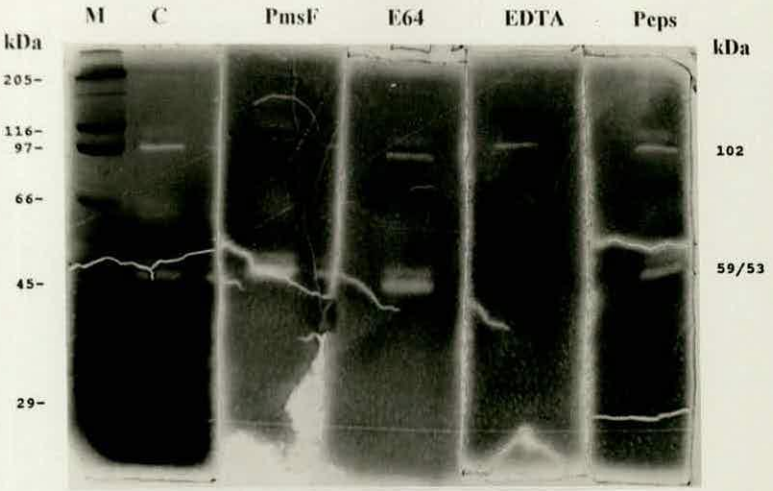
The molecular sizes of adult *T. vitrinus* ES proteinases were estimated by gelatin-substrate gel analysis (section 2.9.9). Following electrophoresis of the ES (2-10 μg protein/track), the SDS was removed from the gel by washing with 2.5% Triton X-100 and the gel was incubated overnight in buffer of appropriate pH, at 37°C. Proteolytic activity was visualised as clear zones on a blue background after staining by Coomassie blue.

ES samples were incubated with the appropriate proteinase inhibitor prior to electrophoresis and, following electrophoresis, the gels were divided into individual tracks and each track was incubated overnight in buffer containing the corresponding proteinase inhibitor. Proteolytic activity (gelatin degradation) was visualised as clear zones on a blue background after Coomassie blue counter staining (section 2.9.3).

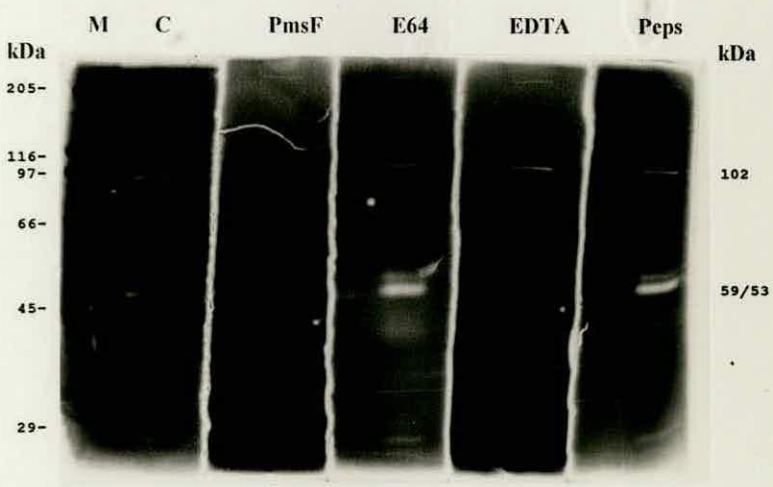
Lanes:

M - high molecular weight protein markers
C - ES only, no proteinase inhibitors
PmsF - serine proteinase inhibitor, phenylmethylsulphonyl fluoride (1 mM)
E64 - cysteine proteinase inhibitor, transepoxysuccinyl-L-leucylamido (4-guanidino)-butane (10 μM)
EDTA - metallo-proteinase inhibitor (10 mM)
Peps - aspartyl proteinase inhibitor, pepstatin (1 μM)

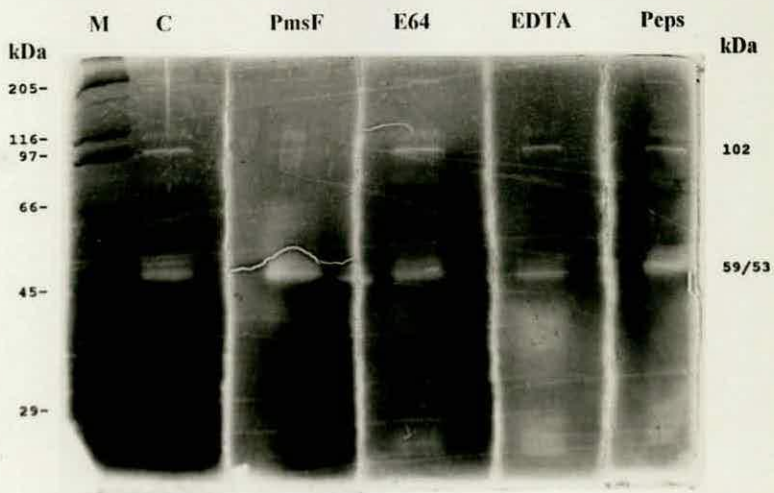
(a) pH 5.5



(b) pH 7.5



(c) pH 9.0



(c) Protein degradation studies

After establishing the presence of proteinase activity in adult *T. vitrinus* ES using the general proteinase substrates, azocaesin and gelatin, the next step was to investigate what type of host proteins may be specifically degraded to give an insight into the possible functions of these secretory proteinases. Commercial preparations of blood proteins were incubated overnight at 37°C with ES material (see section 2.9.11) and the samples analysed by reducing SDS-PAGE (see section 2.9.2). Incubations were carried out at pH 5.5, the optimal pH for proteinase activity (see figure 3.3). The degradation profiles of fibrinogen, plasminogen, albumin and haemoglobin are shown in figure 3.6 (a) and IgG, complement proteins, myosin and fibronectin, in figure 3.6 (b).

Fibrinogen is the precursor of fibrin, a component of the blood clotting process, and consists of 3 pairs of non-identical polypeptide chains linked by disulphide bonds - α (63.5 kDa), β (56 kDa), and γ (47 kDa). Partial proteolysis of the β chain by adult *T. vitrinus* ES is seen in figure 3.6 (a) (fibrinogen, lane C vs. lane *Tv*). Degradation products of this chain of around 45 kDa were also observed in the fibrinogen lane, *Tv*.

Plasminogen, the precursor of plasmin (involved in the degradation of blood clots), is a 72 kDa protein made up of several domains and was completely degraded by the ES proteinases (figure 3.6 (a), plasminogen, lane C vs. lane *Tv*). The two lower bands at approximately 52 and 41 kDa are plasminogen breakdown components due to autoproteolysis. The 52 kDa protein was also degraded by the ES.

Serum albumin (bovine), a carrier protein for a diverse range of substances including membrane lipids, is 60 kDa in size and the red blood cell protein, haemoglobin (14 kDa), which migrates at the level of the dye front, were both unaffected by proteolysis (figure 3.6 (a) albumin and haemoglobin lanes).

IgG consists of heavy (50 kDa) and light (20 kDa) chains, neither of which were degraded by the ES proteinases (figure 3.6 (b) IgG lanes C, vs. lane *Tv*).

Complement is a mixture of about 20 proteins that are involved in many immunological processes. Incubation of the complement mixture with ES resulted in

the degradation of several complement proteins, notably those at approximately 250, 200, 118, 70 and 42 kDa (figure 3.6 (b), arrowed; complement, lane C, vs. lane *Tv*).

The muscle protein, myosin, is made up of two heavy chains at approximately 200 kDa and four light chains of 20 kDa, none of which appeared to be degraded (figure 3.6 (b), myosin, lane C, vs. lane *Tv*).

Fibronectin, a protein associated with connective tissue microfibrils, consists of two polypeptides of 250 kDa (arrowed in figure 3.6 (b), fibronectin, lane C) which were demonstrably cleaved by adult *T. vitrinus* ES, giving breakdown products of approximately 180 and 140 kDa (figure 3.6 (b), fibronectin, lane C, vs. lane *Tv*).

The protein of approximately 60 kDa which appears in most of the control protein tracks was a contaminant (possibly albumin), which was present in the protein preparations used here.

(d) The effect of proteinase inhibitors on plasminogen degradation

From the inhibitor sensitivity profiles, one serine and two metallo-proteinases were identified in the ES products of adult *T. vitrinus*. To determine if either of these proteinase classes was responsible for the degradation of plasminogen, the degradation study was repeated in the presence of the inhibitors, PmsF and EDTA. Figures 3.7 shows the resulting PAGE protein profiles.

The 72 kDa plasminogen protein and the 52 and 41 kDa plasminogen fragments were extensively degraded by the adult *T. vitrinus* ES material (figure 3.7, lane ES). In the presence of the serine proteinase inhibitor, PmSF, this degradation profile was repeated. However, in the presence of the metallo-proteinase inhibitor, EDTA, degradation of the plasminogen was completely inhibited.

The effects of these proteinase inhibitors on the degradation of the other proteins could not be assessed due to the shortage of ES material.

Figure 3.6

The degradation profiles of (a) fibrinogen, plasminogen, albumin and haemoglobin, and (b) IgG, complement, myosin and fibronectin by the ES proteinases of adult *T. vitrinus*.

Commercial preparations of serum proteins were incubated with either buffer alone (C) or adult *T. vitrinus* ES (*Tv*) overnight at 37°C (section, 2.9.11). Incubations were carried out at pH 5.5, the optimum pH for proteinase activity. The samples were analysed by reducing SDS-PAGE and Coomassie blue staining (sections 2.9.2 and 2.9.3). Approximately 7.5 μg/track of complement was loaded. For the other proteins approximately 30 μg/track was loaded onto the gel. The arrows indicate the major protein bands degraded. Track M contains high molecular weight protein markers.

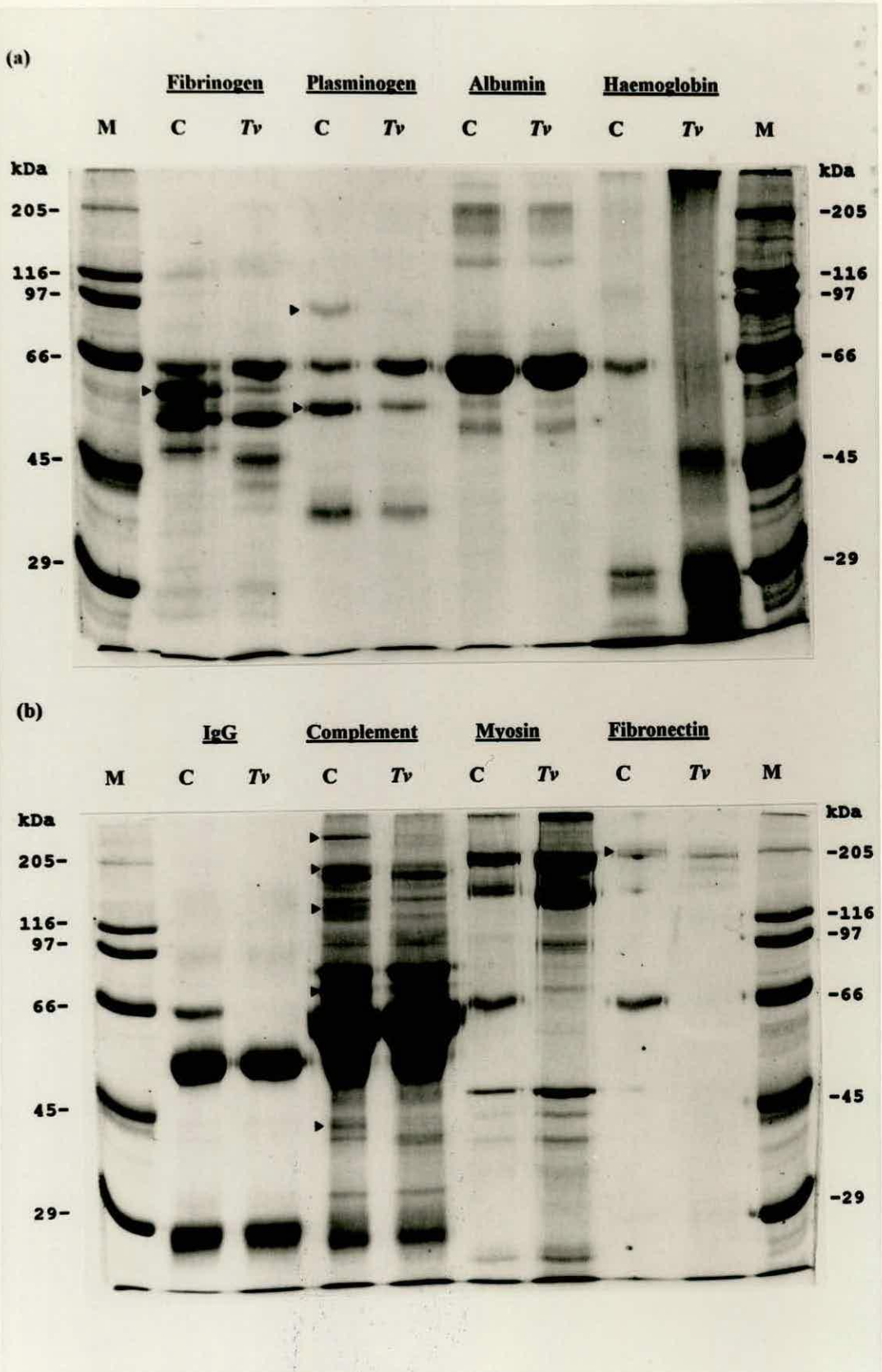
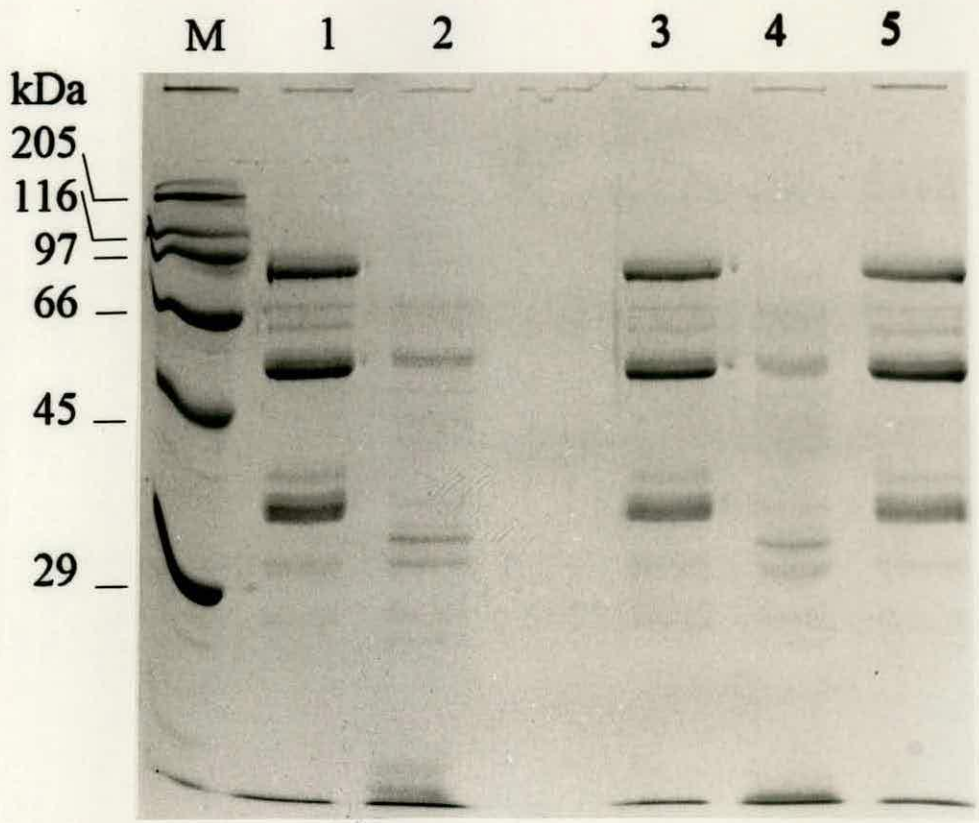

(a)
Fibrinogen
Plasminogen
Albumin
Haemoglobin
M
C
Tv
C
Tv
C
Tv
C
Tv
M
kDa
205-
116-
97-
66-
45-
29-
kDa
-205
-116
-97
-66
-45
-29
(b)
IgG
Complement
Myosin
Fibronectin
M
C
Tv
C
Tv
C
Tv
C
Tv
M
kDa
205-
116-
97-
66-
45-
29-
kDa
-205
-116
-97
-66
-45
-29

Figure 3.7

Effect of proteinase inhibitors on the degradation of plasminogen by adult *T. vitrinus* ES proteinases.

Plasminogen was incubated with either buffer alone (lanes 1 and 3), adult *T. vitrinus* ES (lane 2), adult *T. vitrinus* ES and PmsF (lane 4) or adult *T. vitrinus* and EDTA (lane 5) overnight at 37°C, as described in section 2.9.13. The incubations were carried out in pH 5.5 buffer. The samples were analysed by reducing SDS-PAGE and Coomassie blue staining (sections 2.9.2 and 2.9.3). Each track contains approximately 15 μg of plasminogen. An aliquot of ES only was not analysed as the concentration of ES used in the test samples (approximately 0.5 μg/track) would not be visualised by Coomassie blue staining. Track M contains high molecular weight protein markers.


M
1
2
3
4
5
kDa
205
116
97
66
45
29

3.2.3 Proteinases secreted by L4 *T. vitrinus in vitro* - initial characterisation.

pH optima, gelatin-substrate gel analysis and inhibitor sensitivities

To assess the stage-specificity of *T. vitrinus* ES proteinases, *T. vitrinus* L4 larvae ES was also analysed for proteolytic activity. The amount of concentrated ES material available from the L4 stage of *T. vitrinus* was very small, and therefore proteinase analysis was restricted to defining the pH profile and the mechanistic nature of the enzymes (as determined by gelatin-substrate gel analysis) of ES proteinases on one occasion only.

Proteolytic activity of L4 *T. vitrinus* ES was measured over a range of pH buffers (pH 3.0 - 11.0) as described in section 2.9.8, using azocaesin as substrate. The resultant profile obtained (figure 3.8) showed two main peaks of activity at pH 5.5 and 8.5.

The molecular size and inhibitor sensitivities were then determined, as described for the adult *T. vitrinus* ES proteinases in section 3.2.2 (b). Proteolysis was monitored at pH 5.5 and 8.5 (figure 3.9), the optimal conditions for enzyme activity as determined by the spectrophotomeric assay above.

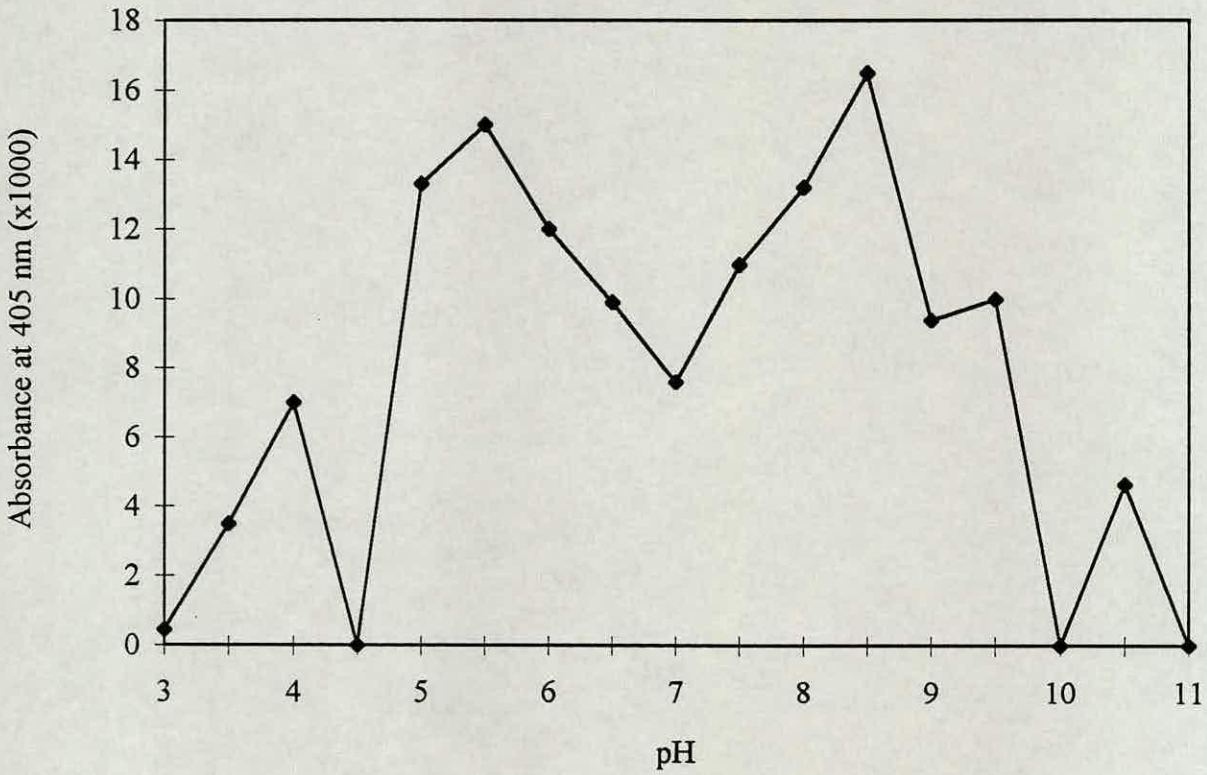
Under acidic conditions, clear bands of proteolysis were evident at approximately 120 and 90 kDa (figure 3.9 (a), lane C), as well as a faint zone of degradation at the top of the gel. PmsF appeared to inhibit the activity of all proteinases present. The cysteine, metallo- and aspartyl proteinase inhibitors, E64, EDTA and pepstatin, respectively, did not inhibit any of the proteolytic enzymes present but, in contrast, seemed to enhance their activity. In the EDTA track, an extra proteinase of approximately 45 kDa was also observed.

At pH 8.5 (figure 3.9 (b), lane C), several proteinases were seen with several bands between 97 and 120 kDa, all of which were inhibited by PmsF, and at least three zones of proteolysis above 205 kDa which were unaffected by any of the inhibitors tested. Although not clearly evident in the figure, a smear of proteolysis was noted in the gel at 40 kDa (see E64 lane) but inhibitor effects were not clearly observed. E64, EDTA and pepstatin had relatively little inhibitory effect on the

proteolysis profile and an additional faint band of proteolysis was seen at about 20 kDa in the presence of E64 and EDTA, although this may have been artifactual because it beyond spread the lateral limits of the tracks in question.

Figure 3.8

pH profile of L4 *T. vitrinus* ES proteinase activity.



The total proteolytic activity of L4 *T. vitrinus* ES (0.2 mg protein/ml) was monitored over a range of pH (pH 3.0 - 11.0) by the method of Knox and Kennedy (1988, see section 2.9.8) using azocaesin as substrate. The pH profile was only determined on one occasion due to the limited ES material available.

Figure 3.9

Gelatin-substrate gel analysis and inhibitor sensitivities of the ES proteinases of L4 *T. vitrinus* at (a) pH 5.5, (b) pH 8.5.

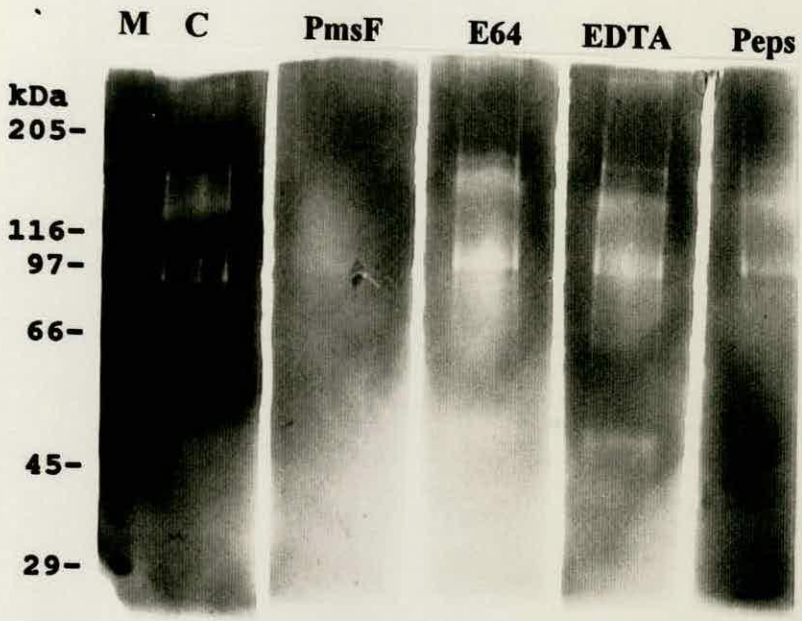
The molecular sizes of L4 *T. vitrinus* ES proteinases were determined by gelatin-substrate gel analysis (section 2.9.9). Following electrophoresis of the ES (2-8 μg protein/track), the SDS was removed from the gel by washing with 2.5% Triton X-100 and incubated overnight in the appropriate pH buffer at 37°C. Proteolytic activity was visualised as clear zones on a blue background after staining by Coomassie blue.

ES samples were incubated with the appropriate proteinase inhibitor prior to electrophoresis and, following electrophoresis, the gels were divided into individual tracks and each track was incubated overnight in buffer containing the corresponding proteinase inhibitor. Proteolytic activity (gelatin degradation) was visualised as clear zones on a blue background after Coomassie blue counter staining (section 2.9.3).

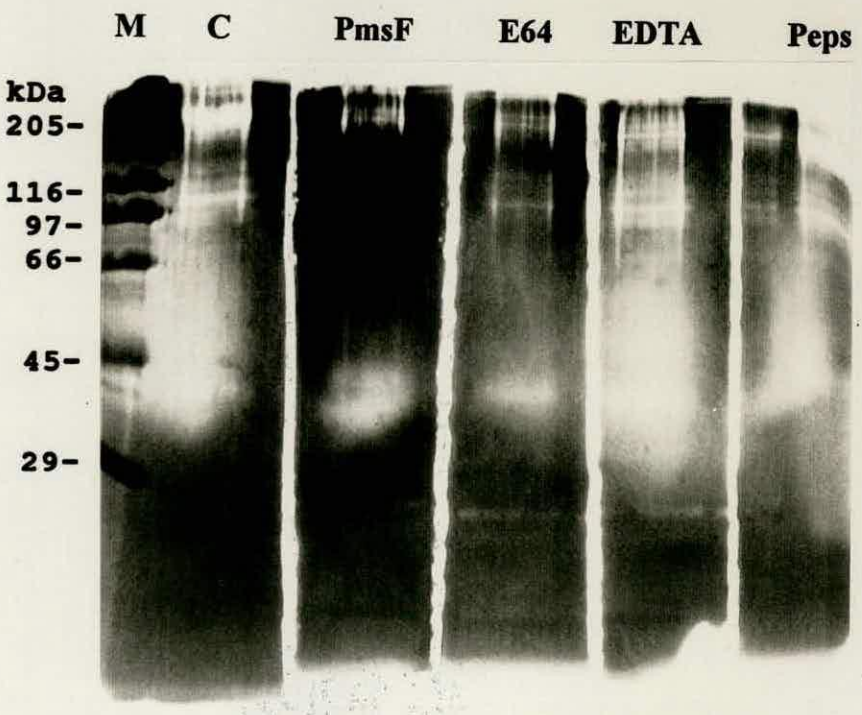
Lanes:

M - high molecular weight protein markers
C - ES only, no proteinase inhibitors
PmsF - serine proteinase inhibitor, phenylmethylsulphonyl fluoride (1 mM)
E64 - cysteine proteinase inhibitor, transepoxysuccinyl-L-leucylamido (4-guanidino)-butane (10 μM)
EDTA - metallo-proteinase inhibitor, EDTA (10 mM)
Peps - aspartyl proteinase inhibitor, pepstatin (1 μM)

(a) pH 5.5


M
C
PmsF
E64
EDTA
Peps
kDa
205-
116-
97-
66-
45-
29-

(b) pH 8.5


M
C
PmsF
E64
EDTA
Peps
kDa
205-
116-
97-
66-
45-
29-

3.3 DISCUSSION

Parasitic nematode secretory enzymes have been ascribed numerous roles in parasitic maintenance within the host and, therefore, may be possible target antigens for vaccine development. The observations of Jones and Knox (1990) and Knox and Jones (1990) revealed, respectively, that *T. vitrinus* secretes AChE and proteinases *in vitro*, but no characterisation of these enzymes was attempted. The results presented here provide a more detailed definition of these activities.

Adult *T. vitrinus* sAChE activity was measured spectrophotometically over a range of pH buffers and optimal activity was recorded at pH 7.5 (figure 3.1). This activity was completely inhibited by the specific AChE inhibitor, eserine, confirming that psuedocholinesterases did not contribute to the activity observed (table 3.1). The overall pH of the mammalian small intestine is approximately neutral to slightly alkaline, therefore, it is appropriate that *T. vitrinus* sAChE has an optimal pH of 7.5 as *in vivo* the enzyme is most likely to be active at, or near to, the intestinal luminal surface.

Analysis of the adult *T. vitrinus* homogenate by PAGE under native conditions revealed the presence of 2 sharply defined esterases, the upper band being AChE (figure 3.2). A single defined AChE isoform, which migrated through the gel in the same manner as the homogenate AChE, appeared in the ES sample. Rothwell, Ogilvie and Love (1973) also observed 2 esterases in the L4 and adult homogenate of *T. colubriformis*, only one of which was AChE. Similarly, in the ES of L4 *T. colubriformis*, a corresponding single AChE was seen. This is in contrast to the nematodes *N. brasiliensis* and *D. viviparus* where 5 sAChE isoforms have been identified (Blackburn and Selkirk, 1992; McKeand *et al.*, 1994 a). The *T. vitrinus* AChE was found not to migrate very far into the gel, suggesting that the native enzyme is of a high molecular weight or is in the form of a complex multi-subunit - both types have previously been described in AChEs from parasites. An AChE of approximately 980 kDa was identified in *N. brasilensis* (Watts and Atkins, 1981). However, most active forms of AChE have been of the order of 60 - 100 kDa (Rhoads, 1984). The molecular weights of purified *T. colubriformis* sAChEs have

been determined by gel filtration giving proteins of 30, 80 and 189 kDa (Hogarth-Scott *et al.*, 1973; Griffiths and Pritchard, 1994 a). However, the smaller protein (30 kDa) was assumed to be a breakdown product of the 80 kDa protein and the 189 kDa to be a dimer. Blackburn and Selkirk (1992) visualised *N. brasiliensis* sAChEs, using both non-reducing SDS-PAGE, as well as native conditions, and observed bands of enzyme activity at 74 kDa and 39 kDa. However, no AChE activity was evident with adult *T. vitrinus* ES using the same non-reducing SDS-PAGE conditions, indicating that the enzyme differed from the *N. brasiliensis* activity in being SDS sensitive at the concentration used.

AChE usually exists as a multimer of catalytic subunits. SDS is a dissociating agent which would dissociate these subunits during electrophoresis. Subsequent elution of the SDS, prior to specific enzyme staining may not allow the enzyme to refold in an enzymatically active structure. The observation, here, that *T. vitrinus* sAChE exists as a single isoform and is irreversibly inhibited by SDS treatment, suggests that it is fundamentally different from the AChEs secreted by the related rodent intestinal nematode, *N. brasilensis*.

High levels of AChE were released *in vitro* from the L4 and adult stages of *Trichostrongylus* spp., but not the infective L3 stage (Rothwell, Ogilvie and Love 1973; Knox and Jones, 1990), demonstrating that ES components probably altered to deal with the changing environment encountered during nematode development. The infective larvae penetrate the small intestine at the base of the villi and develop in the subepithelial tunnels causing flattening of the mucosa, villous atrophy and lesions (Taylor and Pearson, 1979 a and b). The L4 and adult stages of the *Trichostrongylus* worms burrow freely within the mucosa of the small intestine and secretion of AChE may inhibit local peristalsis and mucus secretion (as described in section 3.1), reducing the chance of trapping and expulsion of the parasite by these processes (Ogilvie and Jones, 1971; Philipp, 1983). During *T. vitrinus* infestation there is a pronounced infiltration of mast cells, basophils, GLs and eosinophils at the site of infection (Rothwell and Dineen, 1972; Coop, Angus and Sykes, 1979). Histamine release by these cells is controlled by mediators such as ACh. Also, ACh is involved in antibody-dependant cellular cytoxicity (Gale and Zighelboim, 1974), lysosomal

enzyme secretions (Ignarro and Colombo, 1973) and neutrophil chemotaxis (Hill *et al.*, 1975). Interference with immune cellular responses may be a very important mechanism by which the parasite evades the hosts immune attack.

Analysis of adult *T. vitrinus* ES proteolytic activity over a range of pH buffers revealed maximal activity in the acidic region (pH 5.0 - 5.5) with a second lower activity peak at alkaline pH (figure 3.3). Proteolysis at neutral pH was relatively low in comparison. However, analysis of the ES proteinases by gelatin-substrate PAGE showed that, the same proteinases, were apparently active at acidic, neutral and alkaline pHs (figures 3.5 b-d). This difference in the apparent activities of the proteinase at different pHs which was observed using the two techniques may be due to the different protein substrates used in each assay. Knox and Jones (1990) demonstrated that the ES components of adult *T. vitrinus* degraded elastin-orcein far more efficiently than azocasein at pH 7.4. pH affects proteinase activity by altering the enzyme structure as well as by modulating the ionic balance at the active site. pH will have parallel effects on substrate structure and altered protein folding may expose or mask specific proteinase cleavage sites. Though the overall pH optimum for the small intestine is generally neutral/slightly alkaline, the pH of the parasites immediate environment may be considerably affected by the breakdown of host tissue, mucus secretion, the hosts inflammatory response, disruption of the electrolyte and water balance, and leakage of plasma proteins back into the intestine. Also, the degradation of specific proteins may be pH dependant. Therefore, the broad pH range of individual proteinases observed in the gelatin substrate gels may have evolved as an adaptation to suit this changing environment. Equally, these enzymes need not function at the optimal pH for activity.

Gelatin-substrate analysis of the ES revealed the presence of several proteinases, as distinguished by their molecular size, active over a broad pH range (pH 5.5, 7.5 and 9.0, figures 3.4). No correlation could be made between the protein and proteinases profiles, though the dominant protein at approximately 50 kDa (figure 3.4) may correspond to the major 53 kDa proteinase. Inhibition studies revealed the presence of a serine proteinase of approximately 102 kDa and two metallo-proteinases at 53 and 59 kDa. However, the 53/59 kDa doublet was only inhibited by

the metallo-proteinase inhibitor, EDTA, at pH 5.5 and 7.5 but not under alkaline conditions. It is possible that the high pH somehow affected the inhibitory ability of EDTA. No cysteine or aspartyl proteinases were detected. The proteinase inhibitors were added to both the ES samples prior to electrophoresis and also in the overnight buffers. The serine proteinase inhibitor, PmsF, has a short half-life (100 min at 25°C at pH 7.0; Price and Johnson, 1989) however, the inhibitor binds irreversibly to the enzyme.

Initial characterisation of the proteolytic enzymes secreted by the L4 stages of *T. vitrinus in vitro*, indicated that proteinase activity had a biphasic pH profile (pH 5.5 and 8.5), similar to the adult ES profile. Gelatin-substrate gel analysis showed different proteinases were active at acidic and alkaline conditions unlike the observations for adult ES proteinases. At pH 5.5, the main proteolytic activities were at 120 and 100 kDa, and both were inhibited with the serine proteinase inhibitor, PmsF. At pH 8.5, proteinases of >205, and around 100 kDa were dominant: the inhibitor profiles indicated that the 100 kDa activities were due to serine proteinase but the >205 kDa activities could not be classified with the inhibitors used. Like adult ES no cysteine or aspartyl proteinases were present. Proteinases may also have been observed at neutral pH when analysed by gelatin-substrate gels as with the adult stage, but this was not tested due to the limited L4 ES material available. In comparison with the adult, apart from the presence of a similarly sized 100 kDa serine proteinase, the L4 form appears to release a distinctly different set of proteolytic enzymes during *in vitro* culture.

Protein degradation studies demonstrated that adult *T. vitrinus in vitro* ES proteinases are capable of degrading fibrinogen, plasminogen, some complement proteins and fibronectin but did not digest albumin, haemoglobin, myosin or IgG (figures 3.6 (a) and (b)). Soluble fibrinogen is the precursor of insoluble fibrin, a component in the blood clotting cascade. Digestion of the β chain of fibrinogen by ES proteinases would suggest that adult *T. vitrinus* ES possessed an anticoagulant function even though the nematode is not apparently an obligate blood feeder. Fibrinogen degrading proteinases have previously been observed in the ES from the ovine abomasal nematode, *O. circumcincta*, which is also not an obligate blood-feeder

(Young, Knox and McKeand, 1995). Fibrinogen degradation by adult *O. circumcincta* ES was mainly due to metallo-proteinase activities, whereas, the anticoagulant activities of the blood-feeding parasite, *H. contortus*, have been related to cysteine proteinases (Knox *et al.*, 1993).

Plasminogen is the precursor of plasmin, a protein involved in the breakdown of clots. Hotez and Cermani (1983) noted that the digestion of plasminogen by *A. caninum* produced two smaller proteins of 58 and 40 kDa, which were similar to plasminogen products formed by leukocyte elastase digestion. The authors (Hotez and Cermani, 1983) suggested that these products may form mini-plasmin, a protein that is not readily inhibited by α_2-antiplasmin. The degradation of plasminogen by adult *T. vitrinus* ES may yield active plasmin, promoting the degradation of fibrin clots. This process would, therefore, complement fibrinogen degradation. In addition, plasmin is involved in the activation of the complement system, one of the major effector pathways of the inflammatory response. A number of the complement proteins were also found to be degraded by the ES proteinases (figure 3.7 (c)). The three main functions of the complement system are opsonisation of foreign particles, cytolysis of targeted cells and activation of phagocytic cells (Roitt, Brostoff and Male, 1989). Degradation of plasminogen and complement proteins may be a major way by which the nematode, *T. vitrinus*, disables the hosts immune response to infection. This contrasts with the immune evasion mechanism proposed for *S. mansoni* and *F. hepatica*, the proteinases from both of which have been shown to cleave IgG, suggesting that immunoglobulin molecules which are bound to the surface of the parasite may be removed by ES proteinases (Auriault *et al.*, 1981; Chapman and Mitchell, 1982).

T. vitrinus ES proteinases cleaved fibronectin (figure 3.7 (c)), an extracellular glycoprotein which allows cells to interconnect with collagen. The domains of fibronectin can also specifically bind certain molecules outside the cell, such as fibrin, collagen and heparin and so, can act as cell surface receptor (Roitt, Brostoff and Male, 1989). Breakdown of fibronectin may result in the loosening of the tissue network and aid in host tissue invasion.

In agreement with the present study, McKerrow (1989) noted that tissue-penetrating parasite stages secrete mostly serine and metallo-proteinases. Adult *A. caninum* and the pentastomid, *Porocephalus crotali* both secrete alkaline metallo-proteinase which possess elastinolytic properties (Hotez *et al.*, 1985; Jones *et al.*, 1991). The second stage larvae of *Toxocara canis*, which migrate into host tissues, have been reported to secrete proteinases *in vitro* that were predominately serine proteinases and demonstrably degraded components of a model extracellular matrix and basement membranes (Robertson *et al.*, 1989). The L4 *T. vitrinus* stage inhabits the sub-epithelial layers of the intestinal mucosa and the serine proteinases may be involved in the breakdown of the intestinal infrastructure as the worm burrows through the layers. By the time the worm has reached the mature adult stage, the worm is often close to the intestinal lumen, due to the destruction of the intestine surface (Coop, Sykes and Angus, 1979). Proteinase stage-specificity may be a result of the changing structure of the intestinal epithelial layer, varying nutritional needs or exposure to different mechanisms of the hosts immune system.

The research covered in this chapter significantly increases the information available on the AChE and proteinases which are secreted *in vitro* by adult *T. vitrinus*. The release of these enzymes *in vivo* is currently only speculative, but given their proposed importance in establishment and maintenance of the nematode within the host's small intestine, they may be targets of the host immune system and therefore, for candidate antigens for vaccine development. In the future, host antibody (from both serum and intestinal lymph) responses to AChE and the proteinases from adult *T. vitrinus* ES could be assessed.

Initial studies on the *T. vitrinus* L4 stage suggest that proteinase release is stage-specific. It would be interesting to characterise *T. vitrinus* L4 proteinases further and also, those secreted by the L3 stage, to obtain an overall view of the proteinases released throughout the development of the nematode.

Chapter four

Attempts to isolate adult *T. vitrinus* cDNA fragments encoding acetylcholinesterase using the polymerase chain reaction and specific oligonucleotide primers

4.1 INTRODUCTION

4.1.1 General introduction

As described in chapter three, AChE is actively secreted by adult and fourth stage larval *T. vitrinus* during *in vitro* culture, and has been assigned several fundamental roles in maintenance of nematodes within the host (reviewed by Philipp, 1983; Rhoads, 1984; Pritchard, 1993 b). Given the potential importance of sAChE to the survival of *T. vitrinus* within the host, the enzyme may be a possible target of the host's immune response. Indeed, lambs which are immune to *T. vitrinus* express antibody responses that recognise several adult parasite ES components (chapter three). To assess the host protective ability of sAChE, it must first be purified. Recently, Griffiths and Pritchard (1994 a) have successfully isolated sAChE from adult *T. colubriformis* cultured *in vitro*, using edrophonium chloride linked to epoxy-activated sepharose, and the purified sAChE was subsequently used in vaccine trials (Griffiths and Pritchard, 1994 b). However, prior to the start of the work described here, attempts to purify sAChE from adult *T. vitrinus* using edrophonium columns did not seem practical due to the large quantity of ES material required and the very poor yields of enzyme obtained (personal communication, Dr. D.P. Knox, Moredun Research Institute, Edinburgh, UK). Isolation of an antigen directly from *in vitro* cultured ovine nematodes in sufficient quantities to produce a single vaccine dose has been estimated to require three donor sheep (Emery, McClure and Wagland, 1993). Advances in the molecular biology field over the last two decades have now made it possible to produce proteins in large enough quantities for vaccination purposes using recombinant DNA techniques.

Adult *T. vitrinus*, which are readily harvested from donor lambs, secrete AChE in copious quantities and it can be anticipated that the parasite stage should contain abundant AChE encoding mRNA. Because the enzyme is recognised by antibody from infected hosts (Rothwell and Merritt, 1974) it seemed feasible to isolate DNA fragments encoding the enzyme, by immunoscreening cDNA libraries prepared from the adult parasite. However, the antibody responses stimulated during the course of natural infection are weak. Furthermore, suitably discriminating antibody

would be required to identify AChE encoding immunopositives. For the reasons outlined above, antibody probe development was anticipated to be fraught with difficulties and an attractive alternative was to directly isolate DNA fragments encoding AChE by PCR using oligonucleotide primers directed to highly conserved regions of the enzyme molecule pool (Sakanari *et al.*, 1989) and an adult *T. vitrinus* cDNA. Analysis of these fragments should reveal AChE encoding recombinants and such fragments could then be used to re-screen the cDNA library, by hybridisation, for full length clones.

The work detailed in this chapter describes attempts to isolate cDNA fragments encoding AChE from adult *T. vitrinus* using PCR and degenerate oligonucleotide primers. The primers were designed using highly conserved regions of other eukaryotic AChEs for which nucleic acid sequence was available. The same technique has previously been applied successfully in the isolation of other parasite gene sequences, such as genomic DNA sequences encoding serine proteinases from the nematodes, *Anisakis simplex* and *C. elegans*, and the protozoan, *Trypanosoma cruzi* (Sakanari *et al.*, 1989). For the present study cDNA rather than genomic DNA was chosen as the PCR template to avoid the potential complication from introns which might exist in the genomic sequence. Also, since *E. coli* cells are unable to process introns in eukaryotic genes, cDNA would be required for expression of the gene product in these bacterial cells.

4.1.2 Alignment of AChE sequences

At the commencement of this work in November 1991, no nematode AChE gene sequences had been determined. However, AChE genes had been isolated from several mammalian sources, namely human (Soreq *et al.*, 1990), mouse (Rachinsky, 1990), bovine (incomplete sequence from foetal bovine serum; Doctor *et al.*, 1989), *Torpedo californica* (Schumacher *et al.*, 1990) and *Torpedo marmorata* (Sikorav, Krejci and Massoulie, 1987; Sikorav *et al.*, 1988) ray fish, and *Drosophila melanogaster* (Hall and Spierer, 1986). Alignment of the amino acid sequences predicted from these AChE gene sequences indicated regions of high homology throughout the molecule (figure 4.1). Human AChE shows 92%, 54% and 27%

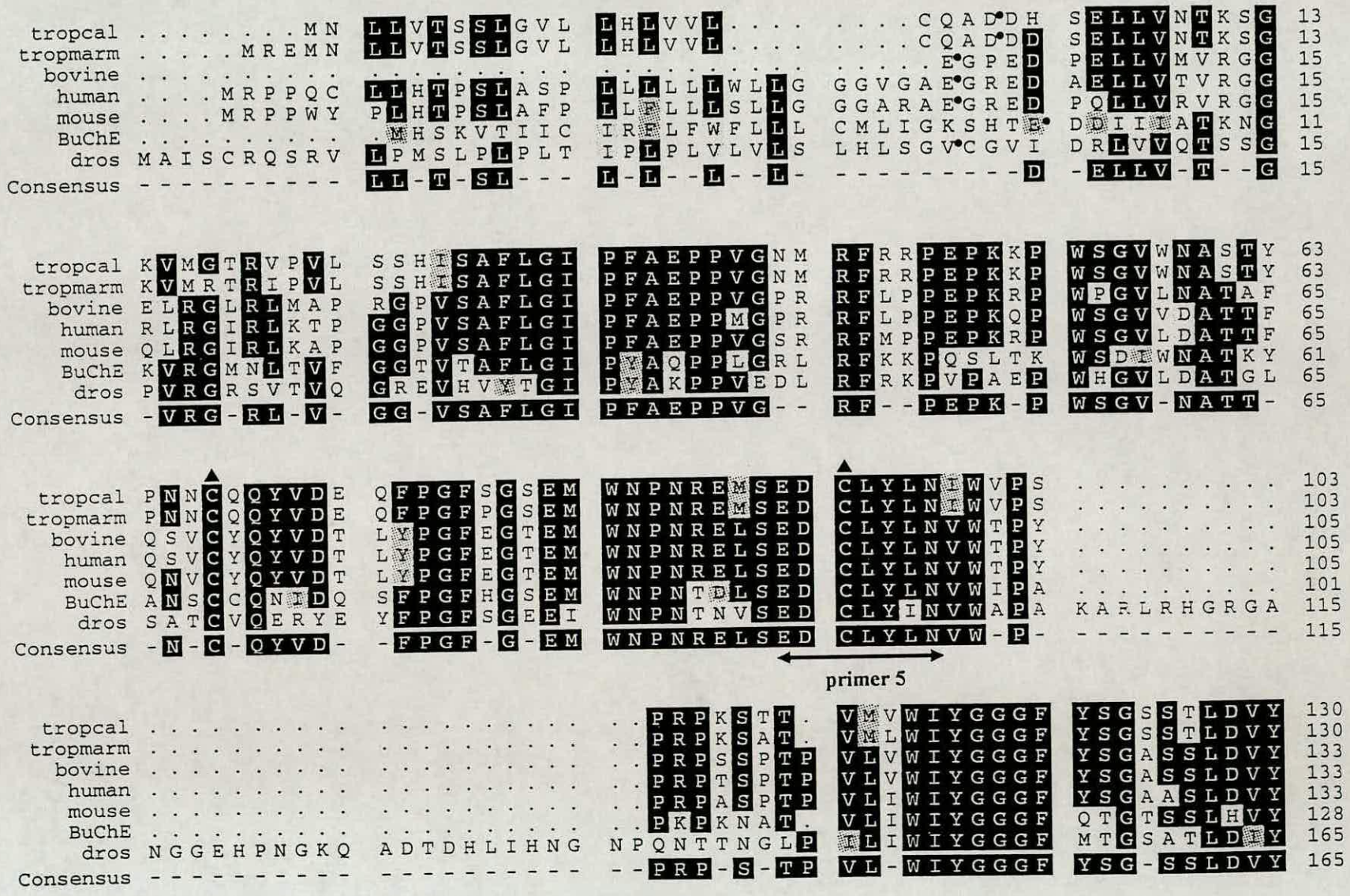
amino acid identity with bovine, *T. californica* and *D. melanogaster* AChEs, respectively. Human AChE also has considerable homology (50%) to human butyrylcholinesterase (BuChE, Soreq *et al.*, 1990).

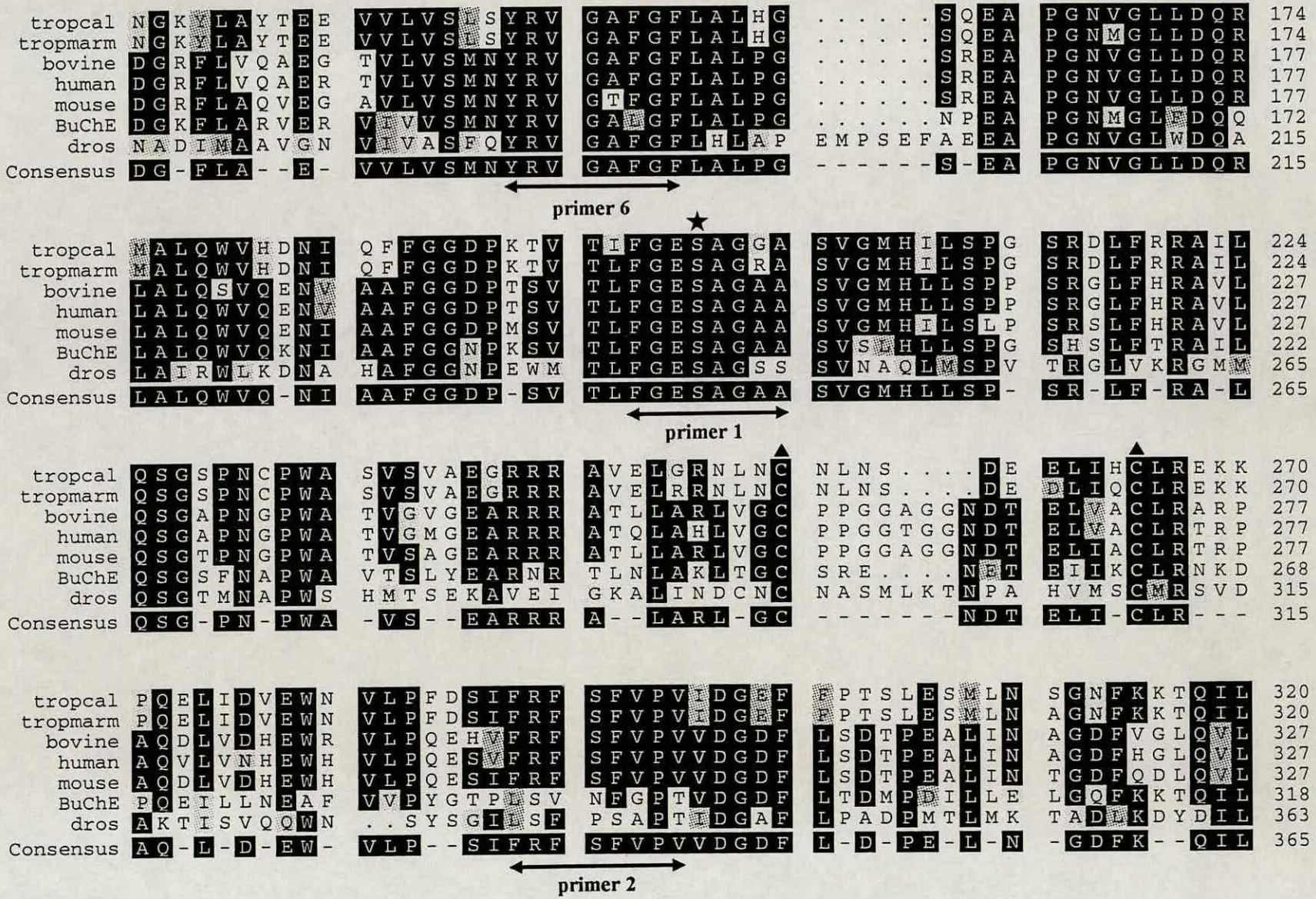
The catalytic mechanism of esterases has not been defined but some features of the human AChE are known. The active site residues, Ser203, His447 and Glu332 have been implicated in the formation of a catalytic triad mechanism (Gibney *et al.*, 1990; Duval *et al.*, 1992; reviewed by Massoulie *et al.*, 1993). Furthermore, three intracatenary disulphide loops are predicted; Cys69-Cys96, Cys257-Cys272, Cys409-Cys529. A fourth disulphide bridge, involving Cys580, has been shown to result in the covalent attachment to the parallel cysteine residue of an identical subunit (Soreq *et al.*, 1990).

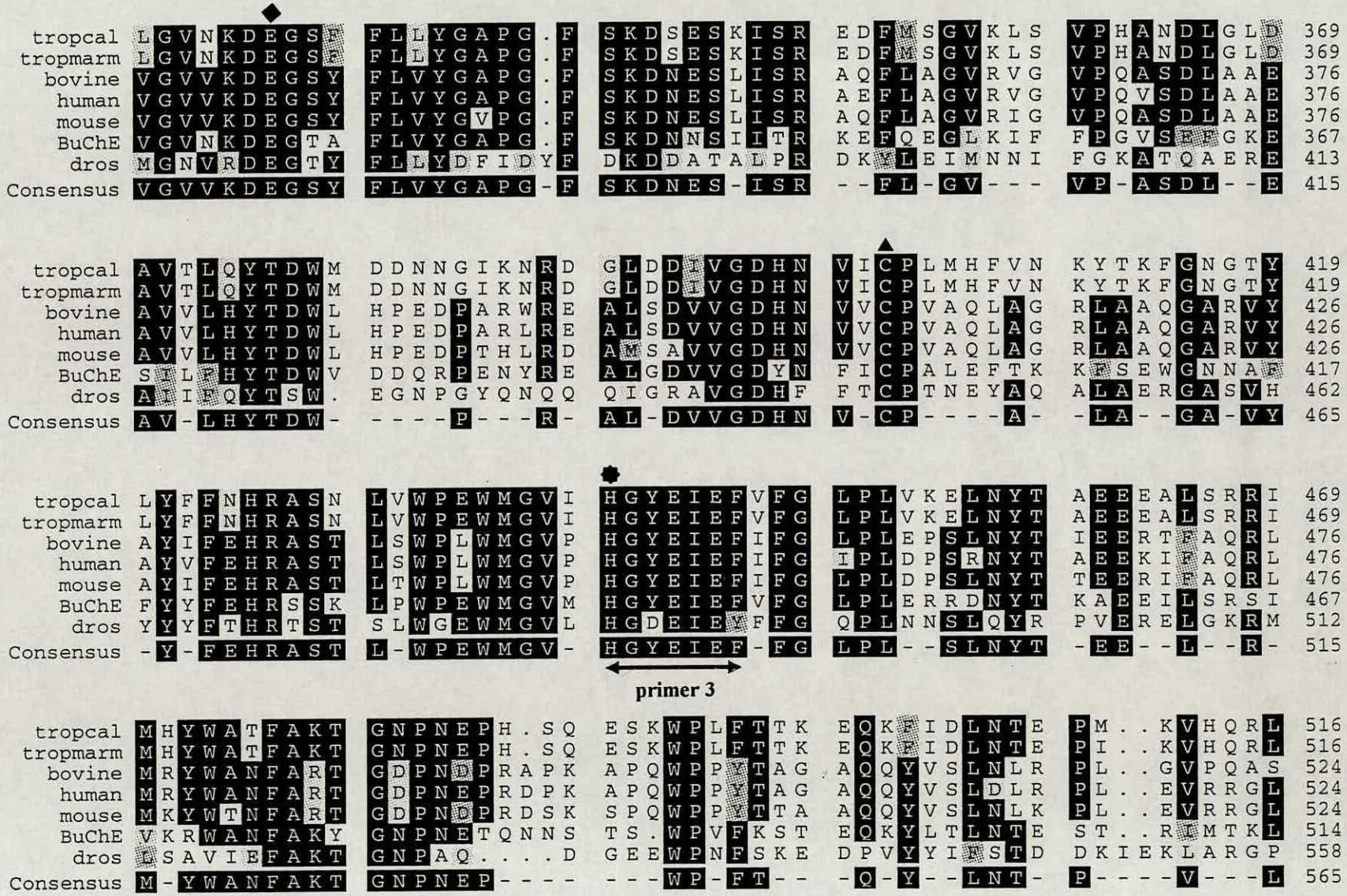
Figure 4.1

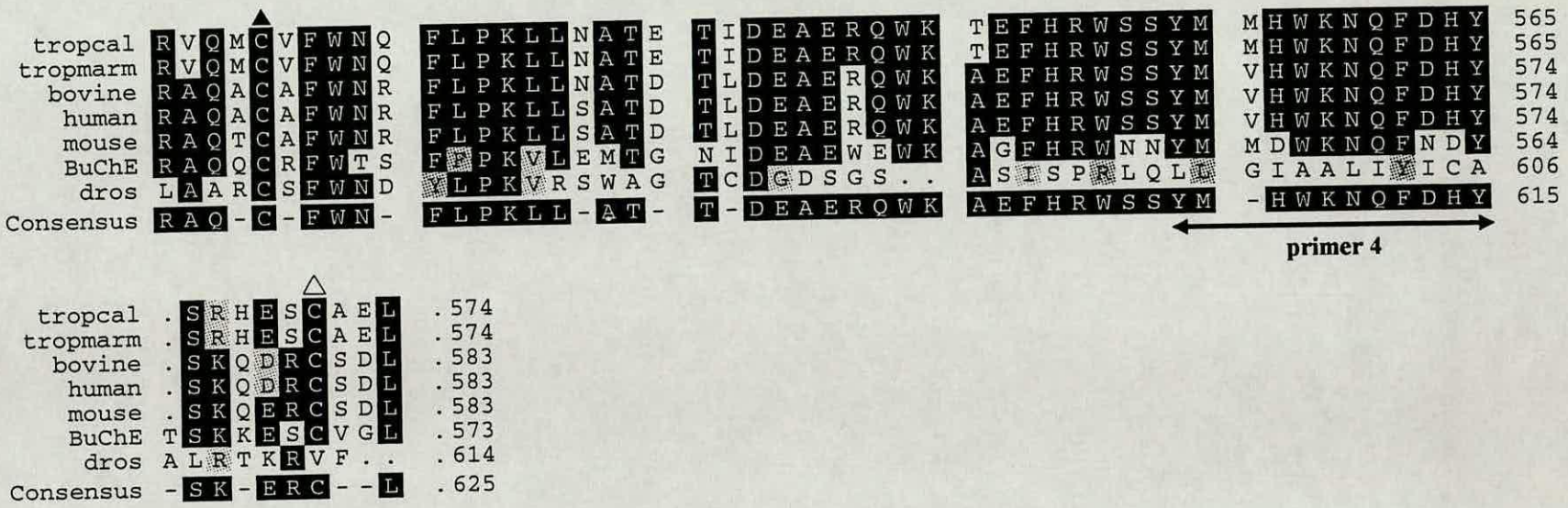
Multiple peptide sequence alignment of AChE.

Comparison of AChE peptide sequences of *Torpedo californica* (torpcal, Maulet *et al.*, 1987), *Torpedo marmorata* (torpmarm, Sikorav, Krejci and Massoulie, 1987,), bovine (Doctor *et al.*, 1989), human (Soreq *et al.*, 1990), mouse (Rachinsky, 1990) and *D. melanogaster* (dros, Hall and Spierer, 1986) and butyrylcholinesterase (BuChE, McTiernan *et al.*, 1987). The amino acid numbering corresponds to the mature protein and the first residue in the mature protein is marked on the right hand side with a dot (•). Identical (■), highly conserved (▩) and conserved (▒) amino acids are shown. A consensus amino acid sequence for AChE based on these sequences is also shown. The following residues are marked: the active site serine (★); the putative His (⬟) and Glu (◆) residues involved in the catalytic triad; the Cys residues involved in intracatenary disulphide bonds (▲); and the Cys residue forming a disulphide bridge with an identical unit (△). The AChE primer sequences (primers 1-6) are indicated by arrows (⟷) (see table 4.1).


tropcal
tropmarm
bovine
human
mouse
BuChE
dros
Consensus
primer 5


tropcal NGKYLAYTEE VVLVSLSYRV GAFGFLALHG .....SQEA PGNVGLLDQR 174
tropmarm NGKYLAYTEE VVLVSLSYRV GAFGFLALHG .....SQEA PGNMGLLDQR 174
bovine DGRFLVQAEG TVLVSMNYRV GAFGFLALPG .....SREA PGNVGLLDQR 177
human DGRFLVQAER TVLVSMNYRV GAFGFLALPG .....SREA PGNVGLLDQR 177
mouse DGRFLAQVEG AVLVSMNYRV GTFGFLALPG .....SREA PGNVGLLDQR 177
BuChE DGKFLARVER VIVVSMNYRV GALGFLALPG .....NPEA PGNMGLFDQQ 172
dros NADIMAAVGN VIVASFQYRV GAFGFLHLAP EMPSEFAEEA PGNVGLWDQA 215
Consensus DG-FLA--E- VVLVSMNYRV GAFGFLALPG -------S-EA PGNVGLLDQR 215
primer 6
tropcal MALQWVHDNI QFFGGDPKTV TIFGESAGGA SVGMHILSPG SRDLFRRAIL 224
tropmarm MALQWVHDNI QFFGGDPKTV TLFGESAGRA SVGMHILSPG SRDLFRRAIL 224
bovine LALQSVQENV AAFGGDPTSV TLFGESAGAA SVGMHLLSPP SRGLFHRAVL 227
human LALQWVQENV AAFGGDPTSV TLFGESAGAA SVGMHLLSPP SRGLFHRAVL 227
mouse LALQWVQENI AAFGGDPMSV TLFGESAGAA SVGMHILSLP SRSLFHRAVL 227
BuChE LALQWVQKNI AAFGGNPKSV TLFGESAGAA SVSLHLLSPG SHSLFTRAIL 222
dros LAIRWLKDNA HAFGGNPEWM TLFGESAGSS SVNAQLMSPV TRGLVKRGMM 265
Consensus LALQWVQ-NI AAFGGDP-SV TLFGESAGAA SVGMHLLSP- SR-LF-RA-L 265
primer 1
tropcal QSGSPNCPWA SVSVAEGRRR AVELGRNLNC NLNS....DE ELIHCLREKK 270
tropmarm QSGSPNCPWA SVSVAEGRRR AVELRRNLNC NLNS....DE DLIQCLREKK 270
bovine QSGAPNGPWA TVGVGEARRR ATLLARLVGC PPGGAGGNDT ELVACLRARP 277
human QSGAPNGPWA TVGMGEARRR ATQLAHLVGC PPGGTGGNDT ELVACLRTRP 277
mouse QSGTPNGPWA TVSAGEARRR ATLLARLVGC PPGGAGGNDT ELIACLRTRP 277
BuChE QSGSFNAPWA VTSLYEARNR TLNLAKLTGC SRE....NET EIIKCLRNKD 268
dros QSGTMNAPWS HMTSEKAVEI GKALINDCNC NASMLKTNPA HVMSCMRSVD 315
Consensus QSG-PN-PWA -VS--EARRR A--LARL-GC -------NDT ELI-CLR--- 315
tropcal PQELIDVEWN VLPFDSIFRF SFVPVIDGEF FPTSLESMLN SGNFKKTQIL 320
tropmarm PQELIDVEWN VLPFDSIFRF SFVPVIDGEF FPTSLESMLN AGNFKKTQIL 320
bovine AQDLVDHEWR VLPQEHVFRF SFVPVVDGDF LSDTPEALIN AGDFVGLQVL 327
human AQVLVNHEWH VLPQESVFRF SFVPVVDGDF LSDTPEALIN AGDFHGLQVL 327
mouse AQDLVDHEWH VLPQESIFRF SFVPVVDGDF LSDTPEALIN TGDFQDLQVL 327
BuChE PQEILLNEAF VVPYGTPLSV NFGPTVDGDF LTDMPDILLE LGQFKKTQIL 318
dros AKTISVQQWN ..SYSGILSF PSAPTIDGAF LPADPMTLMK TADLKDYDIL 363
Consensus AQ-L-D-EW- VLP--SIFRF SFVPVVDGDF L-D-PE-L-N -GDFK--QIL 365
primer 2


tropcal
tropmarm
bovine
human
mouse
BuChE
dros
Consensus
primer 3


tropcal RVQMCVFWNQ FLPKLLNATE TIDEAERQWK TEFHRWSSYM MHWKNQFDHY 565
tropmarm RVQMCVFWNQ FLPKLLNATE TIDEAERQWK TEFHRWSSYM MHWKNQFDHY 565
bovine RAQACAFWNR FLPKLLNATD TLDEAERQWK AEFHRWSSYM VHWKNQFDHY 574
human RAQACAFWNR FLPKLLSATD TLDEAERQWK AEFHRWSSYM VHWKNQFDHY 574
mouse RAQTCAFWNR FLPKLLSATD TLDEAERQWK AEFHRWSSYM VHWKNQFDHY 574
BuChE RAQQCRFWTS FRPKVLEMTG NIDEAEWEWK AGFHRWNNYM MDWKNQFNDY 564
dros LAARCSFWND YLPKVRSWAG TCDGDSGS.. ASISPRLQLL GIAALIYICA 606
Consensus RAQ-C-FWN- FLPKLL-AT- T-DEAERQWK AEFHRWSSYM -HWKNQFDHY 615
primer 4
tropcal .SRHESCAEL .574
tropmarm .SRHESCAEL .574
bovine .SKQDRCSDL .583
human .SKQDRCSDL .583
mouse .SKQERCSDL .583
BuChE TSKKESCVGL .573
dros ALRTKRVF.. .614
Consensus -SK-ERC--L .625

4.1.3 Design of degenerate oligonucleotide primers

Table 4.1 shows the oligonucleotide primers that were designed towards the AChE molecule. Few *Trichostrongylus* spp. gene sequences have been identified, therefore, amino acid codon usage within *Trichostrongylus* spp. could not be defined with useful accuracy. Where there were two possible codons for an amino acid, the codon used in the human AChE was selected. For amino acids with four possible codons, an inosine was placed in the third base position. For amino acids possessing six possible codons, the most common first two bases of the codon were chosen and an inosine was placed in the third position.

Initially, oligonucleotide primers for PCR were designed, based on regions of amino acid identity and conservation (underlined, figure 4.1) and designated primers AChE 1 (sense), AChE 2 (sense and antisense), AChE 3 (sense and antisense) and AChE 4 (antisense) (see table 4.1). Primer AChE 1 was directed towards the region surrounding the active site serine that would be expected to be highly conserved throughout species (see figure 4.1). There are six possible codons for serine, TCN (where N = A or C or G or T) and AGY (where Y = C or T). The active site serine is encoded for by TCG in *D. melanogaster* and by AGT in the *Torpedo* ray and mammalian AChE sequences, therefore, two oligonucleotides were designed towards the primer AChE 1 region, incorporating both possible codons for the active site serine. The difference in active site codons between the invertebrate AChE and the vertebrate AChEs suggested that they evolved separately, as a single point mutation would be insufficient to convey the difference directly (Massoulie *et al.*, 1993).

Primers AChE 2-4 were based on other highly conserved amino acid regions of the AChE molecule distal to the active site serine. The primer sequence for AChE 2 encodes the phenylalanine residues, Phe295 and Phe297, two of fourteen aromatic residues that line the active site gorge of AChE (Sussman *et al.*, 1991). The first amino acid encoded for in primer AChE 3 is His447 and this amino acid is thought to be involved in the putative catalytic triad mechanism (Gibney *et al.*, 1990). The peptide sequence encoded for by primer AChE 3 is conserved throughout all the 6 AChEs and BuChE, while the peptide encoded by the primer AChE 2 sequence is not present in the *D. melanogaster* gene or BuChE. The amino acid sequence to which

primer AChE 4 was directed is only conserved within the mammalian AChE (human, bovine and mouse) and the asymmetric form in *Torpedo* spp., and is only partially conserved in BuChE.

Following preliminary PCR reactions, additional primers were designed. An antisense primer to the AChE 1 region was synthesised and was three amino acids longer than the sense AChE1 primer. Primers AChE 5 (sense and antisense) and AChE 6 (sense and antisense) were designed on highly conserved regions in all 6 AChEs proximal to the active site region. AChE 6 region contains one of the six cysteine residues involved in the disulphide bonding.

To amplify the 3' region of the gene, the AChE primers (in sense orientation) were used in conjunction with an antisense primer (dT primer) directed towards the poly A^+ tail of the mRNA (table 4.1). Amplification of the 5' region was attempted using a sense primer directed towards the *trans*-splice leader 1 (SL1) sequence (table 2.2). SL1 is a 22 bp consensus sequence which has been identified 5' to the start codon in a number of nematode mRNAs (reviewed by Nilsen, 1993), including three out of four *C. elegans* actin genes (Kruase and Hirsch, 1987). SLI has also been used to amplify SOD and aspartate proteinase-encoding cDNAs from the ovine GI nematode, *H. contortus* (Dr. S. Liddell and Dr. D.P. Knox, Moredun Research Institute, Edinburgh, UK; personal communication), which is closely related to *T. vitrinus*. A second *trans*-spliced leader sequence (SL2) has also been identified in *C. elegans* (Nilsen, 1989; Huang and Hirsch, 1989). SL2 has yet to be shown in other nematodes (Nilsen, 1989). The exact function of nematode SL sequences has not been defined.

Table 4.1

Degenerate oligonucleotide primers directed towards conserved regions of the AChE molecule.

AChE primer	**Degenerate oligonucleotide primers** upper: nucleotide sequence lower: *corresponding amino acid sequence (in human AChE)*	**position of amino acid residues in human AChE** (sense/antisense)
1	5' TTT GGI GAG AGI/TCI GCI GGI GC 3' *F G E S* A G A*	**200-206** (sense)
	3' TGI GAI AAG CCI CTC AGI CGI CCI CGI CG 5' *T L F G E S* A G A A*	**198-207** (antisense)
2	5' TTI CGI TTI TCI TTI GTI CCI GT 3' *F R F S F V P V*	**296-303** (sense)
	3' AAI GCI AAI AGI AAI CAI GGI CAI 5' *F R F S F V P V*	**296-303** (antisense)
3	5' CAI GGI TAI GAI ATI GAI TT 3' *H G Y E I E F*	**447-453** (sense)
	3' GTI CCI ATI CTI TAI CTI AAI 5' *H G Y E I E F*	**457-453** (antisense)
4	3' GTA GTG GTC GAA CTG GTT CTT CCA GTG *Y M V H W K N Q F* IAC CAT GTA 5' *D H Y*	**562-574** (antisense)
5	5' GAA GAT TGT CTI TAC CTI AA 3' *E D C L Y L N*	**94-100** (sense)
	3' CTT CTA ACA GAI ATG GAI TTA 5' *E D C L Y L N*	**94-100** (antisense)
6	5' TAC CGI GTI GGA GCI TTC GGA TT 3' *Y R V G A F G F*	**151-158** (sense)
	3'ATG GCI CAI CCT CGI AAC CCT AAG 5' *Y R V G A F G F*	**151-158** (antisense)

Nucleotide bases: I = inosine **Amino acids: *S** = active site serine**

Note:

Antisense primers were all synthesised as the inverse complement of the coding DNA sequence.

4.1.4 PCR conditions

Low stringency conditions were applied (low annealing temperature) for the PCRs because of the uncertain homology of *T. vitrinus* AChE(s) to the higher eukaryotic AChEs on which the AChE primers were based. High stringency conditions might actually have selected against the amplification of AChE cDNA fragments if the homology of the primers to the *T. vitrinus* AChE(s) was poor.

The expected sizes of PCR amplified fragments using the AChE directed primers, based on human, *T. californica* and *D. melanogaster* AChE sequences are detailed in table 4.2.

Table 4.2

Expected sizes of PCR amplification cDNA products using AChE primers.

AChE primers	Expected size of amplified cDNA fragment (bp), based on		
	human AChE (full length 1,842 bp)	*T. californica* AChE (full length 1,758 bp)	*Drosophila* AChE (full length 1,947 bp)
1 → 2	321	297	-
1 → 3	774	747	738
1 → 4	1,140	-	-
1 → dT	1,164 +	1,104 +	1,128 +
2 → 3	474	474	-
2 → 4	840	-	-
2 → dT	864 +	831 +	-
3 → 4	381	-	-
3 → dT	405 +	375 +	408+
SL1 → 5	393 +	357 +	405 +
SL1 → 6	567 +	525 +	675 +
SL1 → 1	699 +	675 +	840 +
SL 1 → 2	999 +	951 +	-
5 → 6	192	186	288
5 → 1	324	336	453
5 → 2	624	612	-
6 → 1	153	171	186
6 → 2	453	447	-

4.2 RESULTS

4.2.1 Primary PCR amplification of adult *T. vitrinus* cDNA using oligonucleotide primers directed towards AChE.

Adult *T. vitrinus* (obtained from lambs 14 days p.i.) cDNA (figure 4.2) was prepared from extracted mRNA, as described in section 2.11.6. The cDNA was evident as a smear ranging in size from 300 bp to greater than 2000 bp with the bulk in the range 500 to 1636 bp, of sufficient size to potentially contain full-length AChE encoding cDNAs (see table 4.2). This cDNA was used as the template DNA for PCR amplifications (Saiki *et al.*, 1986; section 2.11.7) using primers AChE 1, AChE 2, AChE 3, AChE 4 and dT. The annealing temperature was 25°C. The resultant amplification products were analysed by 0.8% (w/v) agarose gel electrophoresis (section 2.11.8) and are shown in figure 4.3. In all amplifications described, the first mentioned primer was the sense primer.

PCR with primers AChE 1 and 2 (figure 4.3, lane 1) resulted in the amplification of a 230 bp fragment which was of the approximate size predicted from sequenced AChEs (see table 4.2). This band was successfully amplified from two different preparations of cDNA up to an annealing temperature of 35°C. This was subsequently subcloned into a plasmid vector, the sequence was determined and its identity to AChE assessed (see section 4.2.2).

Amplification of the cDNA using primers AChE 2 and dT produced two dominant fragments of approximately 450 and 240 bp, with the expected size being 800 - 900 bp (figure 4.3, lane 4). These products were Southern blotted and probed under low stringency conditions with ^{32}P end-labelled primer AChE 3 (method described in section 2.11.15), as an internal probe, but no hybridisation was observed. The labelled primer did, however, hybridise to itself (result not shown).

PCR using primers AChE 2 and 3, AChE 3 and poly dT (figure 4.3, lanes 2 and 3 respectively), AChE 1 and 3, AChE 1 and poly dT and, AChE 1 and 4 (results not shown) failed to yield any distinct products.

Figure 4.2

DNA-PAGE profile of adult *T. vitrinus* cDNA preparation.

Adult *T. vitrinus* cDNA was prepared as described in section 2.11.6. An aliquot (1/10) of the cDNA preparation was analysed by PAGE (7.5% [w/v] acrylamide; section 2.11.10) and silver stained for DNA (section 2.11.11).

Lanes:

cDNA - adult *T. vitrinus* cDNA
M - DNA markers

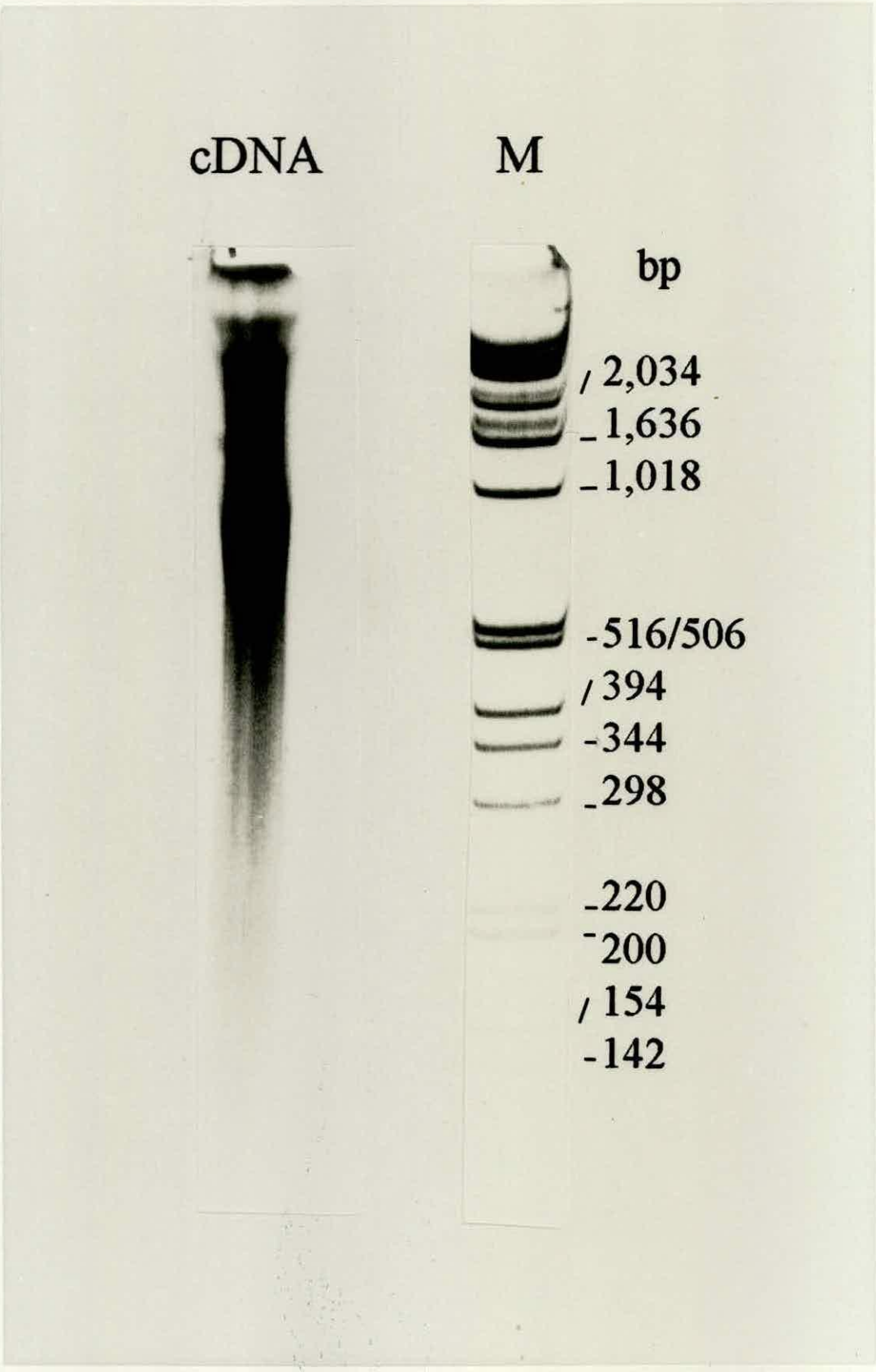

cDNA
M
bp
2,034
1,636
1,018
516/506
394
344
298
220
200
154
142

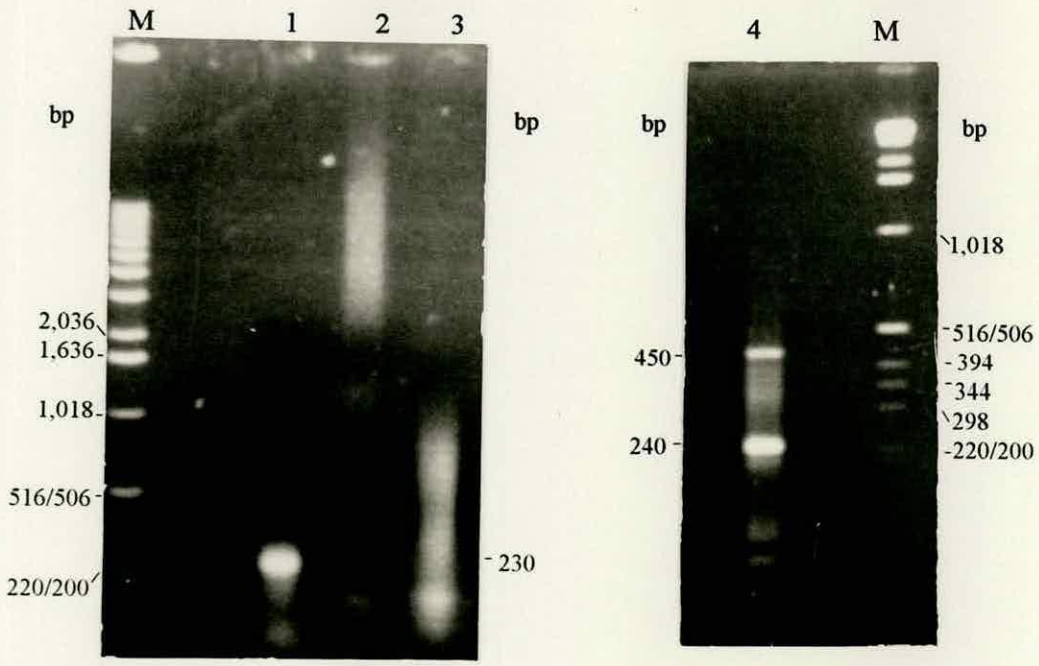
Figure 4.3

PCR amplification of adult *T. vitrinus* cDNA using AChE directed oligonucleotide primers - preliminary results.

PCR amplification of adult *T. vitrinus* cDNA was carried out using AChE directed oligonucleotide primers annealed at 25°C. Products were visualised by agarose (0.8%, w/v) gel electrophoresis. The primers sequences are detailed in table 4.1 and were directed to the regions of the AChE molecule shown in figure 4.1.

Lanes (size markers and primers involved in PCR):

M DNA markers
1 AChE 1 to 2
2 AChE 2 to 3
3 AChE 3 to dT
4 AChE 2 to dT


M
1
2
3
bp
bp
2,036
1,636
1,018
516/506
220/200
230
4
M
bp
bp
1,018
516/506
394
344
298
220/200
450
240

4.2.2 Subcloning of the 230 bp fragment which was amplified using PCR and primers AChE 1 and 2.

The 230 bp fragment amplified by PCR, using primers AChE 1 and 2 (as described in the previous section) was subcloned into the plasmid vector, pCR 1000, (Invitrogen) as detailed in section 2.10.6. Following the transformation step, twenty putative recombinant white colonies and two presumed non-recombinant blue colonies were recovered. Plasmid DNA from each was extracted by the alkaline lysis method (section 2.11.14) and an *EcoR1/HindIII* double restriction enzyme digest was performed on the plasmid DNA (section 2.11.13) to excise the DNA inserts. The expected size of the inserted DNA was approx. 280 bp (230 bp subcloned fragment plus an additional 47 bp from plasmid arms). From the restriction digests it was found that none of the plasmid DNAs isolated from the 20 white colonies (expected to be recombinants) contained DNA inserts. However, one of the blue colonies (B2, not expected to contain an insert) contained plasmid DNA harbouring a DNA insert of, approx. 280 bp, the expected size of the amplified region (figure 4.4).

To confirm that the B2 insert derived from the original 230 bp fragment, a further PCR amplification, again using primers AChE 1 (sense) and 2 (antisense), was carried out on the purified plasmid DNA with an annealing temperature of 35°C. The resultant amplified reaction was analysed by 7.5% (w/v) DNA-PAGE and silver staining (section 2.11.10 and 2.11.11) and a main band was visualised at approximately 230 bp (figure 4.5). A fainter band at 500 bp was also seen.

Figure 4.4

Excision of the 230 bp DNA insert from the recombinant pCR 1000 plasmid vector.

The 230 bp insert was excised by restriction with *EcoR1* and *HindIII* from the recombinant (B2) plasmid DNA and the products of this digest were analysed by agarose (0.8%, w/v) gel electrophoresis. The excised insert includes 47 bp of plasmid.

Lanes:

M DNA markers
1 undigested B2 plasmid DNA
2 *EcoR1/HindIII* restricted B2 plasmid DNA

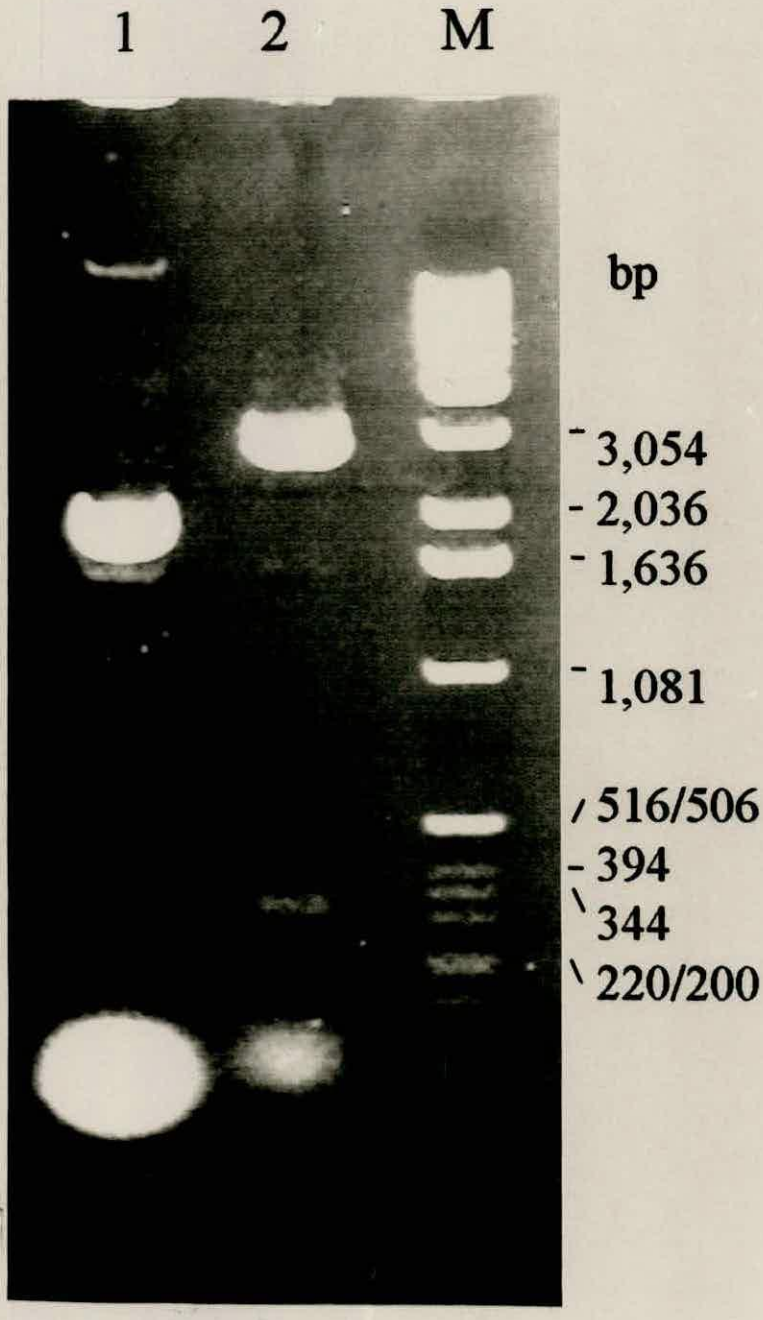

1
2
M
bp
3,054
2,036
1,636
1,081
516/506
394
344
220/200

Figure 4.5

PCR amplification from the B2 plasmid DNA using primers AChE 1 and 2.

The B2 plasmid DNA was subjected to PCR with the oligonucleotide primers AChE 1 and 2 and the products were analysed by DNA-PAGE (7.5% [w/v] acrylamide gel).

Lanes:

M - DNA markers
PCR - amplification from B2 using PCR and primers AChE 1 and 2


M
PCR
bp
516/506
394
298
220/200

4.2.3 Nucleotide sequencing and analysis of the subcloned 230 bp PCR amplified fragment.

The 230 bp fragment was subcloned into the pCR 1000 plasmid vector and sequenced by di-deoxy chain termination (Sanger, Nicklen and Coulson, 1977, section 2.11.15) using the M13 forward and reverse primers (table 2.1) on four occasions. The nucleotide sequence (226 bp) and the deduced amino acid sequence (76 amino acids) is shown in figure 4.6. However, the fragment appeared to have primed at both ends with primer AChE 1 (see figure 4.6).

Analysis of the peptide sequence revealed a continuous open reading frame with significant homology to chicken 60S ribosomal protein L37A (Machida *et al.*, 1993) with 52.7% identity and 84% conservation over 55 amino acids (figure 4.7) and with human 60S ribosomal protein L37A (50.9% identity and 94% conservation over 50 amino acids; Hoof, Fislage and Tuemmler, 1992).

Sequencing of the fragment also showed that 27 bp of extra DNA (TCT CTC GAT ATC GAG TCG ATA TCG AGA) were inserted into the vector at the cloning site, 5' to the start of the 226 bp nucleotide sequence. Following this work, colleagues at the Moredun Research Institute showed that the pCR 1000 vector provided with the Invitrogen TA cloning kit used here did not give a consistent size of 3.0 kb when vector plasmid preparations from individual colonies were restricted with *EcoR1*.

Figure 4.6

Nucleotide sequence and corresponding amino acid sequences for 226 bp PCR fragment amplified with primers AChE 1 and AChE 2.

primer sequence 1

```
5' TTT GGG GAG TCG GCG GGG GCG GCA CTC GAT TAT GGT 36
3' AAA CCC CTC AGC CGC CCC CGC CGT GAG CTA ATA CCA
   F   G   E   S   A   G   A   A   L   D   M   V   12

   CCG TCT TCA GCG TAA AAT GGA CAA AGA AAG ATG GAG 72
   GGC AGA AGT CGC ATT TTA CCT GTT TCT TTC TAC CTC
   R   L   Q   R   K   M   D   K   E   R   W   R   24

   GTA TCC TCA GCA TCC CCG GTA CAC ATT CGT GTT CTG 108
   CAT AGG AGT CGT AGG GGC CAT GTG TAA GCA CAA GAC
   Y   P   Q   H   S   R   Y   T   F   V   F   C   36

   CGC AAA GGA GGC CAT GAA GCG TAA AGC TGT TGG CAT 142
   GCG TTT CCT CCG GTA CTT CGC ATT TCG ACA ACC GTA
   A   K   E   A   M   K   R   K   A   V   G   I   48

   CTG GAA CTG CTC GAA ATA TCA CAA GAG TGT TTC GGT 178
   GAC CTT GAC GAC CTT TAT AGT GTT CTC ACA AAG CCA
   W   N   C   S   K   Y   H   K   S   V   S   G   60

                                          primer
   AGC ATA CGT TTA TGG AAC TGT CAC TGC CGC CCC CGC 214
   TCG TAT GCA AAT ACC TTG ACA GTG ACG GCG GGG GCG
   G   A   Y   V   Y   G   T   V   T   A   A   P   72

   sequence 2
   CCT CTC CCC AAA 3'                             226
   GGA GAG GGG TTT 5
   A   L   S   P                                  76
```

Adult *T. vitrinus* cDNA was amplified using primers AChE 1 (sense) and AChE 2 (antisense) and gave an amplified ~230 bp fragment that was subsequently subcloned into pCR 1000 plasmid vector. The DNA insert was sequenced with M13 forward and reverse primers.

The nucleotide sequence is 226 bp and is shown in codon format while the deduced amino acid sequence is shown in bold. Primer sequence AChE 1 (sense; bases 1-20) is underlined. However, it appears that a second copy of this primer sequence can be found in the antisence strand (bases 207-226) and suggests that AChE 2 was not involved in the amplification reaction.

Figure 4.7

Alignment of the amino acid sequence deduced from the 226 bp PCR fragment with a homologous segment from chicken 60S ribosomal protein L37A.

```
PCR226                          F G E S A G A A L D M V R L Q R 16
Chick60S  A K R T K K V G I V G K Y G T R Y G A S L R 22

PCR226    K M D K E R W R Y P Q H S R Y T F V F C A K 38
Chick60S  K M V K - K I E I S Q H A K Y T C S F C G K 44

PCR226    E A M K R K A V G I W N C S K Y H K S V S G 60
Chick60S  T K M K R K A V G I W H C G S C M K T V A G 66

PCR226    G A Y V Y G T V T A A P A L S P          76
Chick60S  G A W T Y N T T S A V T V K S A I R R L K E 88

Chick60S  L K D Q                                 91
```

Alignment of the amino acid sequences deduced from the 226 bp PCR fragment with chicken 60S ribosomal protein (Chick60S, Machida *et al.*, 1993). Over 55 amino acids (PCR226, residues 16-70 and Chick60S, residues 22-76) there was 52.7% amino acid identity (▨) and 83.6% amino acid conservation (▩).

4.2.4 Secondary PCR amplification of adult *T. vitrinus* cDNA using oligonucleotide primers directed towards AChE.

Subsequent PCR amplifications of adult *T. vitrinus* cDNA were carried out using the various AChE primer combinations detailed in table 4.2, at slightly higher stringency (30°C) and with a fresh cDNA template preparation. The amplification products were analysed by agarose (0.8%, w/v) gel electrophoresis (section 2.11.8). Figure 4.8 shows the resultant amplification profiles using the primers 5' (figure 4.8 a) and 3' (figure 4.8 b) to the active site region. Different cDNA preparations were used in each case. In all amplifications described, the first mentioned primer was the sense primer.

Amplification from SL1 to the active site primer, AChE 1, produced a smearing in lane 3, figure 4.8 (a). Faint banding was observed using SL1 in combination with primers AChE 5, 6 and 2 (figure 4.8 (a), lanes 1, 2 and 4 respectively). PCR using primers AChE 5 and 6 (figure 4.8 (a), lane 5), 5 and 1 (figure 4.8 (a), lane 6) and 5 and 2 (figure 4.8 (a), lane 7) did not give any amplification products. Primers AChE 6 and 1 (figure 4.8 (a), lane 8) produced a dominant band at approximately 1,000 bp, though the expected size was 320-450 (table 4.2). Amplification using primers AChE 5 and 2 (figure 4.8 (a), lane 9) gave two bands of 800 and 250 bp in size, with a product of 600 bp expected (table 4.2). A major DNA band of 500 bp and two fainter fragments at 1,100 and 1,400 were amplified using primers AChE 1 and 2, with no evidence of the 230 bp fragment seen previously in the primary amplifications (figure 4.8 (a), lane 10).

In figure 4.8 (b), PCR using primers AChE 1 and 2 (lane 1) resulted in the repeat amplification of a 500 bp fragment as seen in figure 4.8 (a) (lane 10), but also the appearance of a 400 bp. Additional bands were evident at approximately 420 and 800 bp, though this is not clear from the photograph due to the track being overloaded with the sample. Similarly, primers AChE 1 and 3 gave banding at 800, 500, 420 and 400 bp (figure 4.8 (b), lane 2), though the 500 and 400 bp bands are not as intense as seen in the previous track. The expected size of the product was 750 - 800 bp. Amplification with primer AChE 1 to 4 or to the dT primer (figure 4.8 (b), lanes 3 and 4, receptively) produced faint bands, again in the 420 bp region. PCR

products yielded using primers AChE 2 to 3, or 2 to 4 (figure 4.8 (b), lanes 5 and 6 respectively) appeared as smears, with no evidence of banding. The expected size of the DNA product using primers AChE 2 and dT (figure 4.8 (b), lane 7) was in the region of 850 bp (table 4.2) and several bands were amplified, with the predominant fragments at about 450 and 800 bp. This 450 bp band was seen previously after an amplification using the same primers, though the lower 240 bp fragment (primary amplifications section) was not observed in the present reaction. No amplification of DNA was achieved using primers AChE 3 and 4 (figure 4.8 (b), lane 8), but primer AChE 3 to the dT primer gave banding at approximately 520, 620 and 900 bp.

In summary, PCR of adult *T. vitrinus* using the AChE specific primers amplified several bands which might encode AChE but their identity required confirmation. By this stage, systematic sequencing of a *C. elegans* cDNA library by Waterston *et al.* (1992) had identified three clones that demonstrated significant homology to cholinesterases. Copies of these clones were obtained from Alan Coulson, Cambridge and the cDNA inserts were used to probe the above PCR reactions by Southern blotting in an attempt to identify amplified *T. vitrinus* cholinesterase DNA fragments.

Figure 4.8

PCR amplification of adult *T. vitrinus* cDNA using oligonucleotide primers directed towards AChE - secondary results.

PCR amplification of adult *T. vitrinus* cDNA was carried out using AChE directed oligonucleotide primers annealed at 30°C and visualised by agarose (0.8%, w/v) gel electrophoresis.

(a) PCR using primers 5' to the active site region

Lanes (size markers and primers involved in PCR):

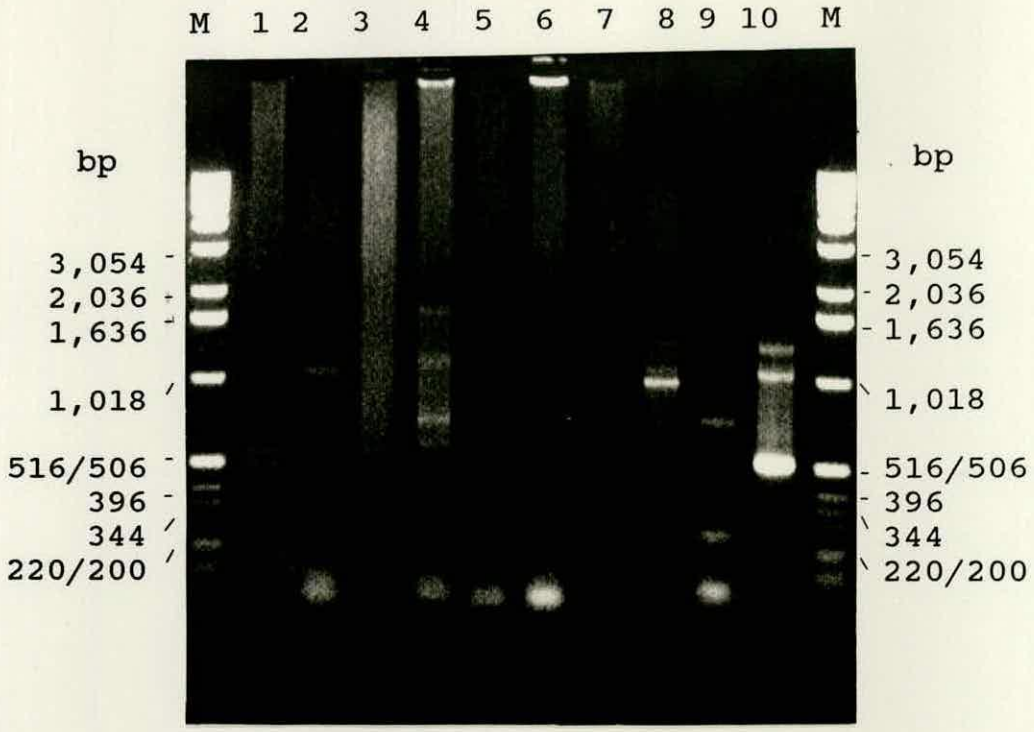
M - DNA markers
1 - SL1 to AChE 5
2 - SL1 to AChE 6
3 - SL1 to AChE 1
4 - SL1 to AChE 2
5 - AChE 5 to 6
6 - AChE 5 to 1
7 - AChE 5 to 2
8 - AChE 6 to 1
9 - AChE 6 to 2
10 - AChE 1 to 2

(b) PCR using primers 3' to the active site region

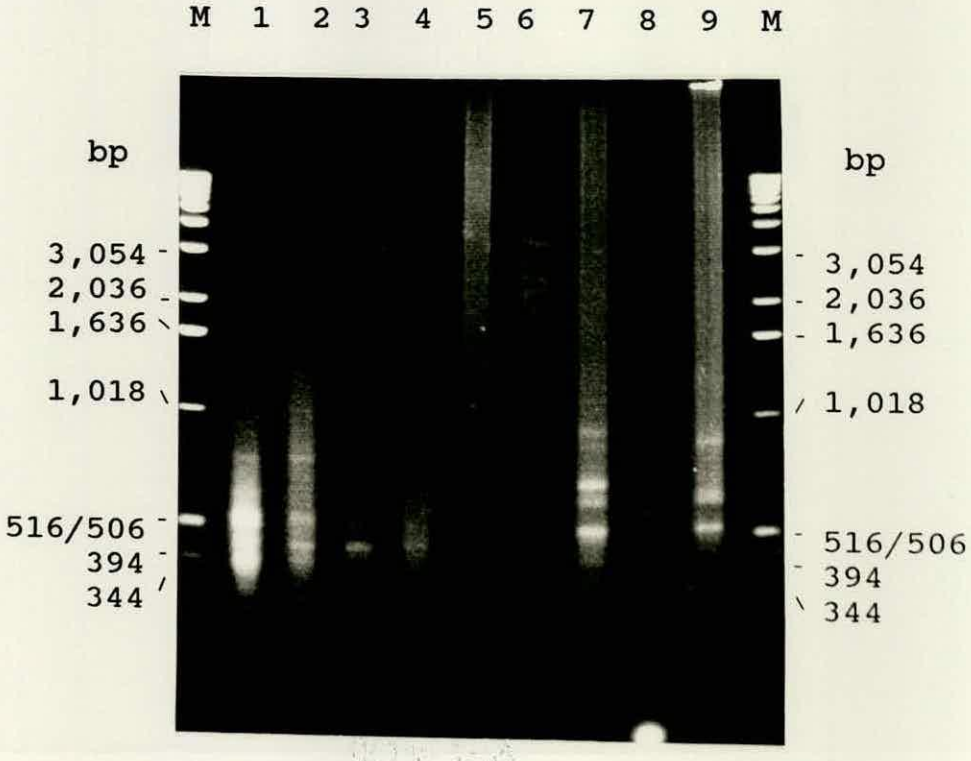
Lanes (size markers and primers involved in PCR):

M - DNA markers
1 - AChE 1 to 2
2 - AChE 1 to 3
3 - AChE 1 to 4
4 - AChE 1 to dT
5 - AChE 2 to 3
6 - AChE 2 to 4
7 - AChE 2 to dT
8 - AChE 3 to 4
9 - AChE 3 to dT

(a)


M 1 2 3 4 5 6 7 8 9 10 M
bp
3,054
2,036
1,636
1,018
516/506
396
344
220/200
bp
3,054
2,036
1,636
1,018
516/506
396
344
220/200

(b)


M 1 2 3 4 5 6 7 8 9 M
bp
3,054
2,036
1,636
1,018
516/506
394
344
bp
3,054
2,036
1,636
1,018
516/506
394
344

4.2.5 Analysis of *C. elegans* cholinesterase clones.

The *C. elegans* cholinesterase clones, cm7d7, cm10g6 and cm06b1 had been sequenced from a single strand reading from the 5' end of each clone and 327, 465 and 503 nucleotide sequence was obtained respectively (Waterston *et al.*, 1992). The first 307 nucleotides of clone cm10g6 was identical to the last 307 nucleotides of cm7d7. All three sequences showed 35-40% amino acid identity to the eukaryotic AChE sequences on which the AChE oligonucelotide primers were based.

To determine the full size of the *C. elegans* DNA inserts, PCR was applied, using the sense SP6 promoter primer and the antisense T7 promoter primer to λSHLX2 (see table 2.2). Two amplifications of each clone were carried out and the products were analysed by agarose (0.8%, w/v) gel electrophoresis (section 2.11.8; figure 4.10).

The amplified cDNA inserts from all three clones were between 1.0-1.6 kb indicating that these amplified products would encode most or all of their respective AChE clones (figure 4.9) by analogy to human, *Torpedo* and *D. melanogaster* AChE nucleotide sequences which are 1,842, 1,758 and 1,947 bp in length respectively.

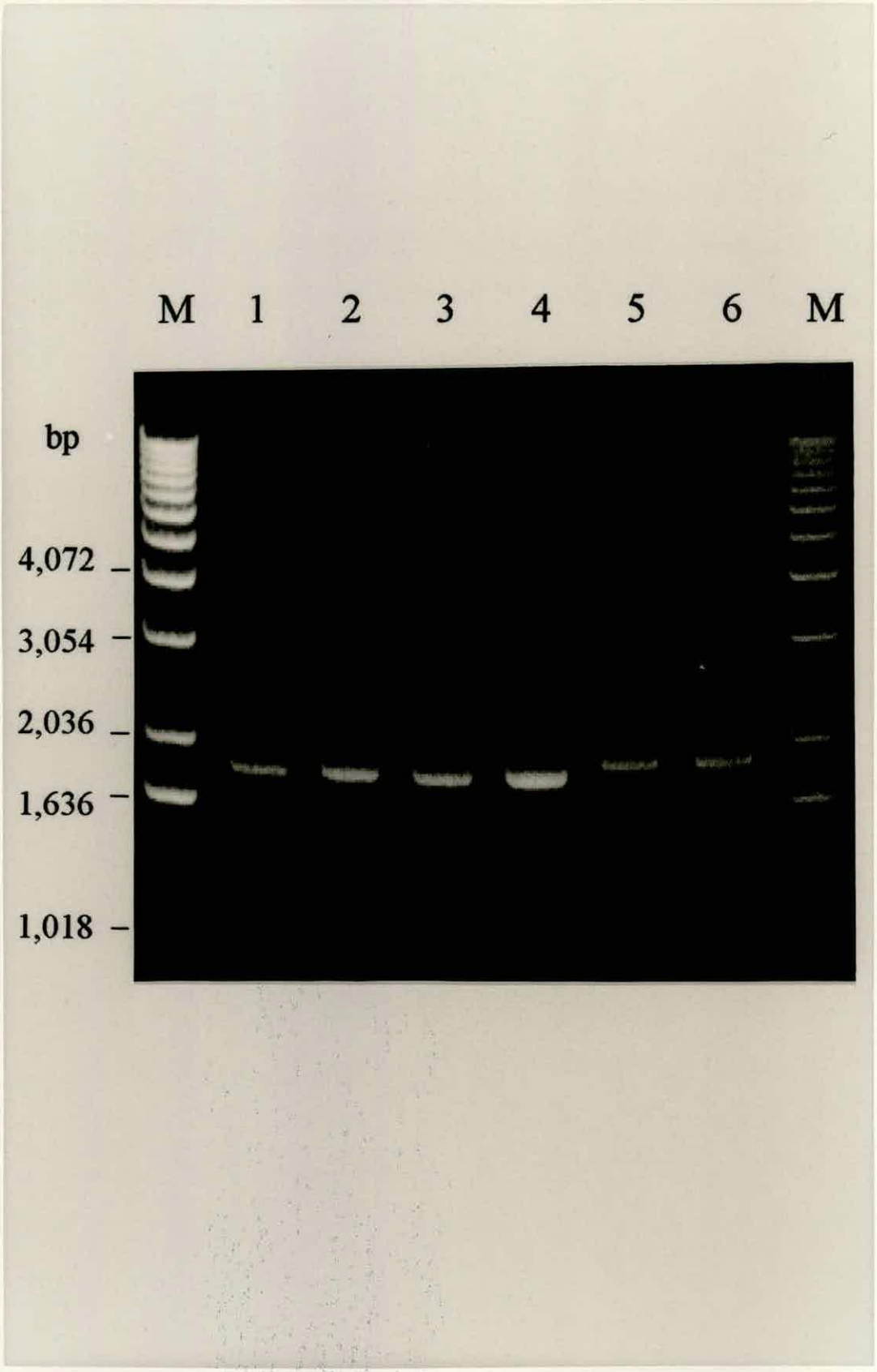
Figure 4.9

Size analysis of the recombinant cDNA inserts of the *C. elegans* clones.

To determine the size of the recombinant cDNA inserts present in the *C. elegans* clones, a PCR amplification was carried out using primers SP6 promoter and T7 promoter present in the λSHLX2 vector and flanking the insertion site. The products were analysed by agarose (0.8%, w/v) gel electrophoresis.

Lanes:

M	DNA markers
1 and **2**	*C. elegans* clone cm7d7 recombinant cDNA insert
3 and **4**	*C. elegans* clone cm10g6 recombinant cDNA insert
5 and **6**	*C. elegans* clone cm06b1 recombinant cDNA insert


M 1 2 3 4 5 6 M
bp
4,072
3,054
2,036
1,636
1,018

4.2.6 Southern blot hybridisation analysis of the PCR products generated with the AChE primers and using ECL-labelled *C. elegans* cholinesterase-encoding cDNA fragments as probes.

Aliquots of the PCR products generated with the AChE primers which were obtained in section 4.2.4 were fractionated by agarose gel electrophoesis (0.8%, w/v) gel electrophoresis (section 2.11.8) and the DNA was transferred by Southern blotting onto nylon membrane (section 2.11.16).

Following fractionation by agarose gel electrophoresis, the amplified cDNA fragments from the *C. elegans* cholinesterase clones were purified using the 'Gene Clean II' kit from Amersham (section 2.11.9). An aliquot of each purified cDNA was electrophoresed on a 0.8% (w/v) agarose gel and Southern blotted onto nylon membrane.

The remaining purified *C. elegans* cholinesterase fragments were ECL random prime labelled, as described in section 2.11.18. The labelled fragments were combined and used to probe the Southern blots prepared above at 42°C (section 2.11.18). The *C. elegans* cholinesterase fragments were used as a mixed probe due to the limited amount of PCR material available as a result of limited cDNA. If a positive result was obtained, the PCR reactions of interest could be repeated and subsequently Southern blotted and probed with individual *C. elegans* cholinesterase fragments. A series of washes (from low to high stringency) was carried out sequentially: 1.0 x SSC containing 0.1% (w/v) SDS at 42°C; 0.5 x SSC containing 0.1% (w/v) at 60°C; 0.2 x SSC containing 0.1% (w/v) SDS at 60°C. After each stringency wash, hybridisation of the labelled probe to the Southern blot was examined. The results are shown in figure 4.10.

Following the primary wash at low stringency (figure 4.10 a), hybridisation of the probe was detected in all of the lanes, with no specific banding evident. With medium stringency washing (figure 4.10 b), hybridisation was restricted to four of the lanes (blot 1, lane 8 and blot 2, lanes 5, 6 and 8). More focal hybridisation was seen in blot 2, lane 5 at approximately 800 and 900 bp, and in lane 6 at 750 bp (figure 4.10 b). After high stringency washing (figure 4.10 c), a smear of DNA stretching from

approximately 1,400 to 400 bp was recognised by the probe in blot 1, lane 8. In blot 2 (figure 4.10 c) banding was evident at about 800 and 900 bp in lane 5.

The ECL labelled *C. elegans* cholinesterase cDNA mixed probe hybridised to the cholinesterase cDNA fragments from the *C. elegans* clones, cm7d7, cm10g6 and cm6b1 as expected and the same profile was observed after each stringency wash (figure 4.11). The positive controls were not placed on the same blot as the test samples as it was not known what the strength of signal from the test samples would be.

Figure 4.10

Southern blots of the PCR amplifications of adult *T. vitrinus* cDNA, using the AChE directed primers, probed with ECL-labelled *C. elegans* cholinesterase cDNA fragments.

The products of the PCR amplifications from adult *T. vitrinus* cDNA, using the AChE directed primers, were separated by agarose gel electrophoresis, Southern blotted onto nylon membranes and probed with ECL-labelled cholinesterase cDNA-combined fragments from *C. elegans*. Following hybridisation at 42°C, the nylon membranes underwent a series of washes at (a) low stringency wash in 1x SSC, containing 0.1% [w/v] SDS at 42°C, (b) medium stringency wash in 0.5x SSC, containing 0.1% [w/v] SDS at 60°C and (c) high stringency wash in 0.2x SSC, containing 0.1% [w/v] SDS at 60°C.

Blot 1 PCR using primers 5' to the active site region (see figure 4.8 (a) for agarose gel profile).

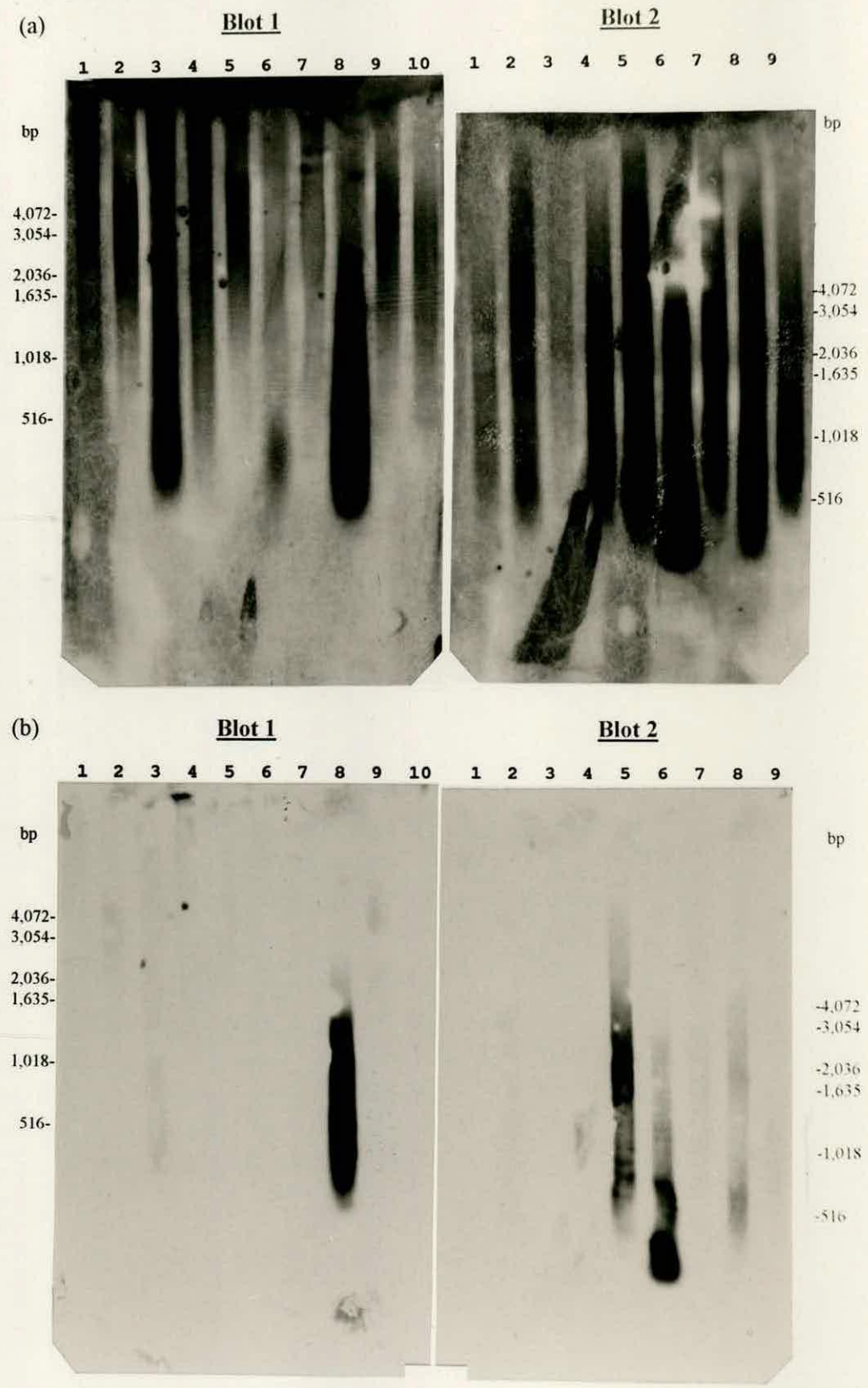
Lanes (size markers and primers involved in PCR):

M - DNA markers
1 - SL1 to AChE 5
2 - SL1 to AChE 6
3 - SL1 to AChE 1
4 - SL1 to AChE 2
5 - AChE 5 to 6
6 - AChE 5 to 1
7 - AChE 5 to 2
8 - AChE 6 to 1
9 - AChE 6 to 2
10 - AChE 1 to 2

Blot 2 PCR using primers 3' to the active site region (see figure

Lanes (size markers and primers involved in PCR):

M - DNA markers
1 - AChE 1 to 2
2 - AChE 1 to 3
3 - AChE 1 to 4
4 - AChE 1 to dT
5 - AChE 2 to 3
6 - AChE 2 to 4
7 - AChE 2 to dT
8 - AChE 3 to 4
9 - AChE 3 to dT


(a)
Blot 1
1 2 3 4 5 6 7 8 9 10
bp
4,072-
3,054-
2,036-
1,635-
1,018-
516-
Blot 2
1 2 3 4 5 6 7 8 9
bp
-4,072
-3,054
-2,036
-1,635
-1,018
-516
(b)
Blot 1
1 2 3 4 5 6 7 8 9 10
bp
4,072-
3,054-
2,036-
1,635-
1,018-
516-
Blot 2
1 2 3 4 5 6 7 8 9
bp
-4,072
-3,054
-2,036
-1,635
-1,018
-516

(c)

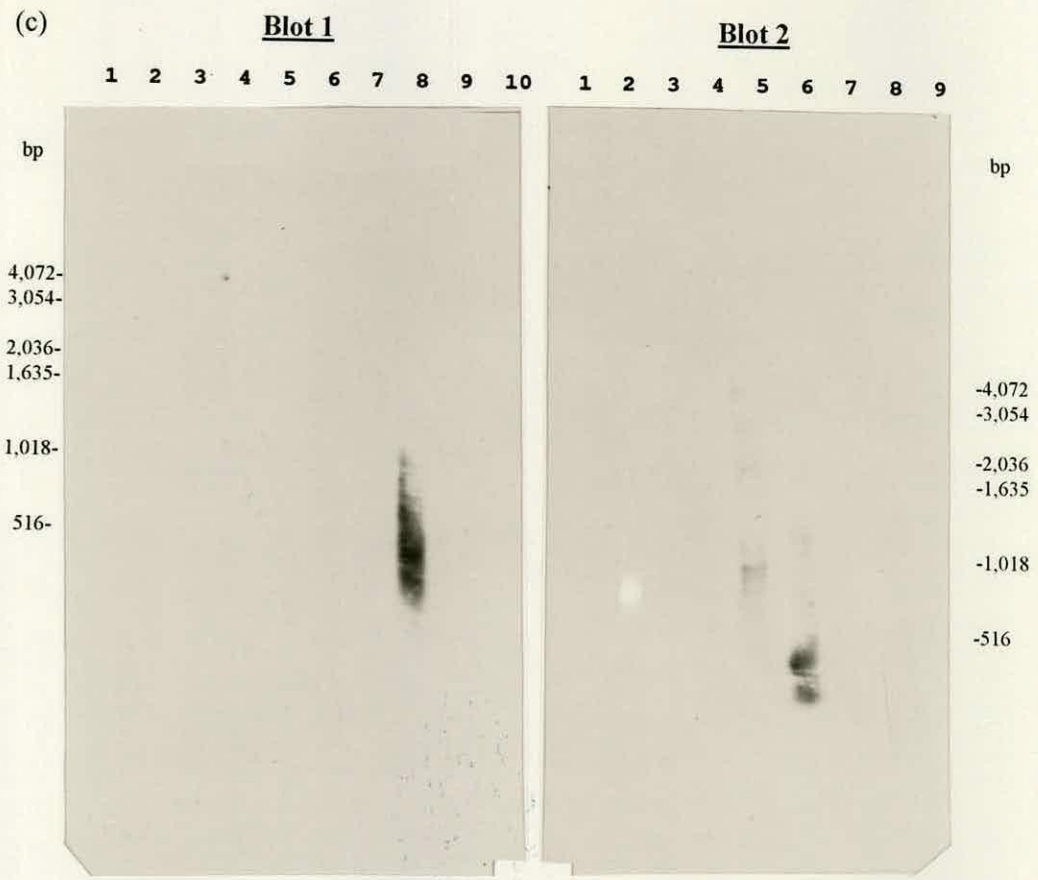

Blot 1
1 2 3 4 5 6 7 8 9 10
bp
4,072-
3,054-
2,036-
1,635-
1,018-
516-
Blot 2
1 2 3 4 5 6 7 8 9
bp
-4,072
-3,054
-2,036
-1,635
-1,018
-516

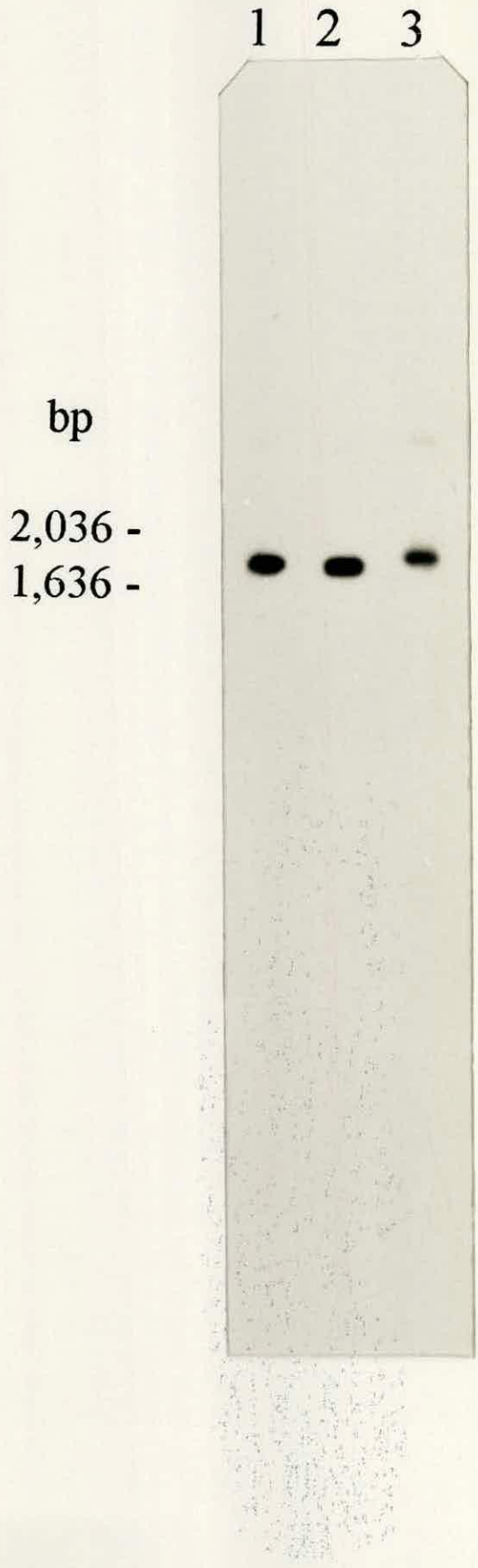
Figure 4.11

Southern blot of *C. elegans* cholinesterase cDNA fragments, probed with ECL-labelled *C. elegans* cholinesterase cDNA fragments.

The PCR amplified cDNA inserts from *C. elegans* clones cm7d7, cm10g6 and cm06b1 were separated by agarose (0.8%, w/v) gel electrophoresis and Southern blotted onto a nylon membrane. Following this, the blot was probed with ECL-labelled cDNA inserts from these clones in unison with the previous blots, to provide a positive control. Shown here is the resultant hybridisation profile after the final stringency wash at 60°C in 0.2 x SSC, containing 0.1% (w/v) SDS.

Lane:

1 *C. elegans* clone cm7d7 recombinant cDNA insert
2 *C. elegans* clone cm10g6 recombinant cDNA insert
3 *C. elegans* clone cm06b1 recombinant cDNA insert


1 2 3
bp
2,036 -
1,636 -

4.3 Discussion

The present chapter, describes attempts to isolate adult *T. vitrinus* cDNA fragments encoding AChE (both secretory and non-secretory) using PCR and specific oligonucleotide primers.

Initial PCR, using primers AChE 1 and 2 and adult *T. vitrinus* cDNA as template at low annealing temperature, resulted in the amplification of a 230 bp fragment (figure 4.3, lane 1) that approximated with the predicted size, as judged by reference to existing cholinesterase amino acid sequences (figure 4.1), The fragment was subcloned into the plasmid vector, pCR 1000 and the nucleotide sequence was determined. The derived peptide sequence had significant homology (57.6% amino acid identity) to chicken and human 60S ribosomal protein, but with no apparent relationship to the cholinesterase family.

The isolation of the recombinant plasmid from *E. coli* which conveyed the ability to hydrolyse X-gal may reflect on the fact that the insert contained, at the 5' end, 27 bp of extra nucleotide sequence inserted into the cloning site. Studies carried out at Moredun Research Institute (personal communication; Dr. S. Liddell) on plasmid pCR 1000 which was used in this study implied that the vector was unstable and prone to rearrangement. Analysis of the sequence of the cloned product revealed the presence of AChE 1 primer sequence at both ends of the amplified 230 bp fragment. This may reflect on the low annealing temperature used. Primer AChE 1 (sense) and AChE 2 (antisense) are 60% similar over 23 nucleotides and under low stringency conditions it may be possible that they are able to anneal to each other.

Amplification from the cDNA using primers AChE 2 and the dT primer (figure 4.3, lane 4) gave two bands of 450 and 240 bp, both of which were below the expected size of 800-900 bp. By analogy to existing cholinesterase sequences (figure 4.1), the highly conserved region encoded by primer AChE 3 should have been present in these PCR products if they did encode a classical cholinesterase. However, when a Southern blot of this PCR reaction was probed with labelled AChE 3, hybridisation was not detected (data not shown), though the probe hybridised to a Southern blot of AChE 3. This result either indicated that the primers used were not sufficiently specific for cholinesterases, that the target cDNA was of poor quality, or

that *T. vitrinus* cholinesterases differ from existing cholinesterase sequences used for primer design (figure 4.1). Moreover, non-specific PCR products may have been due to the low annealing temperature (25°C) used. However, low stringency amplification conditions would be expected to yield products including the target sequence as well as additional non-specific products. The target cDNA ranged in the size from greater than 5000 bp to approx. 300 bp and therefore was of sufficient size to encode cholinesterases by comparison to existing sequences. Furthermore, since the cDNA was prepared from the adult parasite, where the mRNA would be expected to be present in abundance, then an ample proportion of the AChE clones ought to be present in the cDNA pool.

Secondary PCR amplifications of adult *T. vitrinus* cDNA were carried out, using a new cDNA preparation involved various AChE primer combinations, along with the SL1 and dT primers and were performed at slightly higher stringency (30°C). Amplification using primers AChE 1 and 2 on separate occasions gave two different profiles (figure 4.8 (a) lane 10 and (b) lane 1), neither of which amplified the 230 bp fragment seen previously. This confirmed the impression that either one or both primers had insufficient specificity for the target sequence. Alternatively, this may be due to variance in the different cDNA preparations used, although they appeared similar in terms of size and yield. In figure 4.8 (b), the amplification products from PCR using primers AChE 1 to 2 and AChE 1 to 3 were very similar. This suggested that the common bands were produced from annealing of the shared primer, AChE 1, to both ends of the fragments, though amplifications using only AChE 1 as a primer were not carried out.

Increasing the stringency of PCR may have actually selected against the amplification of AChE fragments due to the uncertain homology of adult *T. vitrinus* AChE to AChEs from higher eukaryotes on which the primers were based. Therefore, in an attempt to differentiate cholinesterase-related amplified adult *T. vitrinus* fragments, the PCR products were probed with *C. elegans* cDNA fragments encoding cholinesterases. The *C. elegans* cholinesterase cDNAs were identified by sequencing 300 - 500 nucleotides from a single strand from the 5' end of each clone (Waterston *et al.*, 1992). Computer analysis of the corresponding amino acid

sequences concluded that the *C. elegans* cholinesterase clones were 35-40% identical at the amino acid level to AChEs from higher eukaryotes. The actual size of each clone was determined to be between 1.0 - 1.6 kb (figure 4.9). Human, *Torpedo* and *D. melanogaster* AChE nucleotide sequences are 1,842, 1,758 and 1,947 bp in length respectively, therefore, each *C. elegans* clone appeared to contain a cDNA insert that may encode for most, or all, of their respective cholinesterase genes.

Southern blot analysis showed that in the PCR amplification products using primers AChE 6 and , the *C. elegans* cholinesterase cDNAs hybridised to a smear of DNA ranging from approximately 1,200 to 400 bp in size, with no distinct banding evident (figure 4.10 c, blot 1, lane 8). The corresponding DNA profile (figure 4.8 a, lane 8) showed a dominant band at approximately 1,000 bp with slight smearing below this. The expected size of product for this particular reaction was 320 - 450 bp. The smearing effect obtained may be caused by the annealing of only one of the primers in the PCR reaction to the template cDNA resulting in single stranded products of variable size. *C. elegans* cholinesterase cDNAs also recognised two bands at approximately 800 and 900 bp from the PCR amplification using primers AChE 2 and 3 (figure 4.10 c, blot 2, lane 5). The actual DNA profile for this reaction (figure 4.8 b, lane 5) appeared as a smear, with no discrete banding. In the amplification of adult *T. vitrinus* cDNA with primers, AChE 2 and 4, the *C. elegans* cholinesterase probe hybridised to a smear of DNA in the region of 100 - 300 bp (figure 4.10 c, blot 2, lane 6). Again, the profile of the DNA in this reaction produced a smear when analysed on an agarose gel (figure 4.8 b, lane 6).

The predicted size of amplified AChE cDNA fragments using primers AChE 2 and 3, and AChE 2 and 4 were about 470 and 840 bp respectively. However, in the hybridisation study, the amplified DNA fragments recognised by the *C. elegans* cholinesterase clones in the PCR using primers AChE 2 and 3 were actually larger than the products obtained with the primers AChE 2 and 4. Thus, though the *C. elegans* cholinesterase cDNA fragments hybridised at high stringency, to certain products from the PCR amplification of *T. vitrinus* cDNA using AChE directed primers, no precise conclusions as to the nature of the fragments can be drawn.

Recently, utilising low stringency PCR and oligonucleotide primers identical to primer AChE 5 (sense) and AChE 1 (antisense), Arpagaus *et al.* (1994) isolated a 691 bp fragment from *C. elegans* genomic DNA. Sequencing of the fragment revealed that, in total, 336 bp at the 5' and 3' ends which showed significant homology to previously defined cholinesterase sequences and an internal 355 bp portion which was determined to be intronic were also identified (Arpagaus *et al.*, 1994). In *C. elegans*, three different types of AChE genes (*ace-1*, *ace-2* and *ace-3*) have been found which encode for three different catalytic types of AChE (classes A, B and C respectively) (Johnson *et al.*, 1988). This is in contrast to vertebrates and insect AChE which is encoded by a single gene in each of the sources (Taylor, 1991; Hall and Spierer, 1986). The complete *C. elegans* AChE gene, which included the 691 bp fragment, was isolated by Arpagaus *et al.* (1994) and corresponded to *ace-1*. *Ace*-1 did not hybridise to the other two AChE genes, *ace-2* and *ace-3*, suggesting that the AChE encoded for by the latter two genes are distinctly different to *ace-1* (Arpagaus *et al.*, 1994). The codon for the active site serine in the *ace*-1 gene was TCA, as in *Drosophila*, though overall, the *ace*-1 encoded AChE showed higher identity with those AChEs from vertebrates (Arpagaus *et al.*, 1994). The *ace-1* was found not to be *trans*-spliced with either SL1 or SL2 (Arpagaus *et al.*, 1994).

The AChE amino acid sequences on which the AChE oligonucleotide primers used in the present study were based were almost identical to the corresponding amino acids in *C. elegans ace-1* gene. If *T. vitrinus* contains only AChE gene(s) similar to *ace-2* or *ace-3*, the lack of hybridisation of *ace-1* to *ace-2* or *ace-3* (Arpagaus *et al.*, 1994) would suggest that the use of PCR cloning, based on the oligonucleotide primers which were chosen, may not be possible.

In summary, amplification of adult *T. vitrinus* cDNA fragments encoding AChE, using PCR and specific primers, has not been straightforward. The lack of success with the technique appeared to be due, at least in part, to the low stringency conditions that were applied to the PCRs, dictated by the uncertain homology of *T. vitrinus* AChE to AChEs from the higher eukaryotes. Though a similar procedure has been successful for the amplification of other nematode enzymes, such as serine

proteinase from *A. simplex* (Sakanari *et al.*, 1989), little information is available on studies where the technique was found not to be feasible.

Further investigation of the PCR amplified *T. vitrinus* DNA fragments that were recognised by the *C. elegans* cholinesterase cDNA probes is required but was outwith the time-scale of this study. It would be useful to subclone and sequence the products of the PCR reactions which give positive hybridisation signals with the *C. elegans* probes at high stringency. Although the hybridising bands are not of the expected size, this analysis would allow further definition of these products and, perhaps, the isolation of cholinesterase encoding cDNAs.

Chapter five

Molecular characterisation of adult *T. vitrinus* excretory/secretory components by immunoscreening an adult *T. vitrinus* complementary DNA library with specific antiserum

5.1 INTRODUCTION

ES proteins of parasitic nematodes stimulate protective immune responses in the host (e.g. Rothwell and Love, 1974; O'Donnell *et al.*, 1989 b). ES is made up of a complex mixture of components that are either actively shed from the surface of the parasite, or, are released from specialised excretory-secretory organs (Lightowlers and Rickard, 1988). Adult *T. vitrinus* ES contained at least 18 proteins as judged by non-reducing SDS-PAGE (see figure 3.4) and enzyme analysis demonstrated the presence of AChE and several proteolytic enzymes (see chapter three). SOD activity has also been identified in adult *T. vitrinus* ES (Knox and Jones, 1992). The following work describes the characterisation of other components of the adult *T. vitrinus* ES.

The approach taken was to immunoscreen an expression library of adult *T. vitrinus* cDNA, constructed in the expression vector, λgt11, with antiserum raised against the *in vitro* ES proteins of adult *T. vitrinus*. This is a procedure which has been applied to identify antigen-encoding genes from several other parasites (e.g. Young *et al.*, 1985; Knight *et al.*, 1986; Donelson *et al.*, 1988; Werner *et al.*, 1989). As described in section 2.2.4, in λgt11, foreign DNA is inserted into an *Eco*R1 cloning site in the C-terminal region of the β-galactosidase (*lac*) gene (Young and Davis, 1983 b). The *E. coli* strain, Y1090, acts as the host for propagation and expression of λgt11 cDNA library for immunoscreening, whereas Y1089 cells are used for producing recombinant lysogens permitting larger quantities of the fusion protein to be produced. Inserted eukaryotic DNA is unlikely to possess promoter and ribosomal binding sites which can be recognised by *E. coli* and therefore transcriptional and translational factors from *E. coli* genes must be employed in λgt11. Both *E. coli* strains have several common features. During the initial stages of growth, synthesis of potentially toxic fusion peptides is inhibited by the expression of high levels of a lac repressor (*lac* I gene product) encoded by the plasmid pMC9. *Lac* I promoter repression can be blocked by the addition of IPTG and this, in turn, allows expression of the downstream gene from p*lac*. Expression of the foreign peptide as a translational fusion to the C-terminal of β-galactosidase ensures that the

protein will be produced effectively in *E. coli* and helps to protect it from digestion by intracellular proteases. Potentially, the stability of the protein is also enhanced by the absence of the lon protease from the host strains as a result of mutation of the *lon* gene. Both host *E. coli* strains, Y1089 and Y1090, are r_k^- m_k^+, ensuring the absence of the *Eco* K restriction enzyme, thus, preventing loss of clones that contain target sequences for this enzyme. In addition, Y1089 carries *hfl*A150 to promote lysogenic growth and lacks *sup*F rendering it lysis-defective (Young and Davis, 1983 a).

Expression libraries in λgt11 may be made from genomic DNA or cDNA. With genomic DNA all the genes encoded by the organism are present, providing the library is large enough. However, genomic DNA contains both coding and non-coding regions leading to the requirement to screen more phage plaques in order to find the clone of interest. Also, the coding regions of genomic DNA may be interrupted by introns and, therefore, may not be expressed. In contrast, cDNA originates from mRNA and contains only coding regions. However, the cDNA preparation represents the population of genes expressed only at the time of the mRNA extraction and during the life-cycle of a parasite, protein expression is often stage-specific. In the present study, the aim was to isolate gene fragments encoding ES proteins synthesised by the adult stage of *T. vitrinus*, therefore, it was advantageous to use an expression library constructed from adult stage cDNA library. mRNA isolated from adult *T. vitrinus* should contain a high proportion of mRNAs which transcribe proteins that are actively produced by the parasite at that stage, including proteins that are excreted or secreted.

5.2 RESULTS

5.2.1 Analysis of Western blots of ES with antisera

Two batches of adult *T. vitrinus* ES (ES A and ES B) were separated by SDS-PAGE under reducing conditions, after which, the gels were silver stained for protein (section 2.9.2 and 2.9.3; figure 5.1 (a)). In ES A, four protein bands were evident at approximately 40, 66, 105 and 130 kDa. In ES B, the main protein bands appeared at approximately 66, 60, 55 and 40 kDa. In both ES A and ES B two faint bands above 205 were also seen on the actual gel, though they are not visible in figure 5.1.

Rabbit antibodies to adult *T. vitrinus* ES proteins were raised as described in section 2.8.6. The antiserum was defined on the basis of its recognition of *T. vitrinus* ES proteins (ES A) on Western blot strips (section 2.9.4). A number of proteins that corresponded to the protein profile of ES A were recognised by the anti-adult *T. vitrinus* ES serum (figure 5.1 (b), lanes 1 and 2). There appeared to be little difference between the recognition profile of ES proteins that had been treated with periodate and those that had not (lane 1 compared with lane 2) although the banding on the periodate treated blot was sharper due to reduced background (lane 1). No ES proteins were recognised by normal rabbit serum (lane 3). For immunoscreening purposes, the anti-ES serum was diluted 500-fold.

Western blot strips of adult *T. vitrinus* ES (ES B) were also probed with anti-adult *T. colubriformis* sAChE serum and serum from sheep hyperimmune to *T. vitrinus* infection. The anti-adult *T. colubriformis* sAChE serum was prepared by G. Griffiths, University of Nottingham, Nottingham, UK. The sAChE was purified from *T. colubriformis* ES using edrophonium chloride linked to epoxy-activated Sepharose (Griffiths and Pritchard, 1994 a). SDS-PAGE (non-reducing conditions) analysis of the purified sAChE gave a single band of 66 kDa, as determined by specifically staining the gel for protein and AChE activity (Griffiths and Pritchard, 1994 a). Gel filtration of the purified AChE determined the presence of 80 and 189 kDa proteins, designated as globular monomer and dimer (G1 and G2) forms respectively, and also, an AChE breakdown component of 38 kDa (Griffiths and Pritchard, 1994 a). This

material was injected into a rabbit to raise antiserum against the purified adult *T. colubriformis* sAChE. The proteins recognised by the anti-sAChE serum were concentrated at the top of the Western blot (figure 5.1 (b), lanes 4 and 5) and no proteins were recognised by normal rabbit serum (lanes 6 and 7).

The hyperimmune sheep serum was obtained from Dr. F. Jackson, Moredun Research Institute, Edinburgh, UK. A range of proteins, 30->205 kDa, were recognised by the hyperimmune serum (figure 5.1 (b), lane 8) although the recognition profile was slightly fainter for the periodate treated strip (figure 5.1 (b), lane 9). With the pre-immune sheep serum a few of the proteins recognised by the hyperimmune serum were detected in the non-periodate strip (figure 5.1 (b), lane 10) but no proteins were in the periodate treated track (figure 5.1 (b), lane 11).

Figure 5.1

(a) Reducing SDS-PAGE of adult *T. vitrinus* ES

Adult *T. vitrinus* ES was concentrated then separated (2-10 μg/track) by reducing SDS-PAGE (7.5% [w/v] acrylamide). The gel was silver stained for protein. Two different batches of ES (ES A and ES B) were analysed.

Lanes:

M - protein high molecular weight marker
ES - adult *T. vitrinus* ES concentrated 40-fold

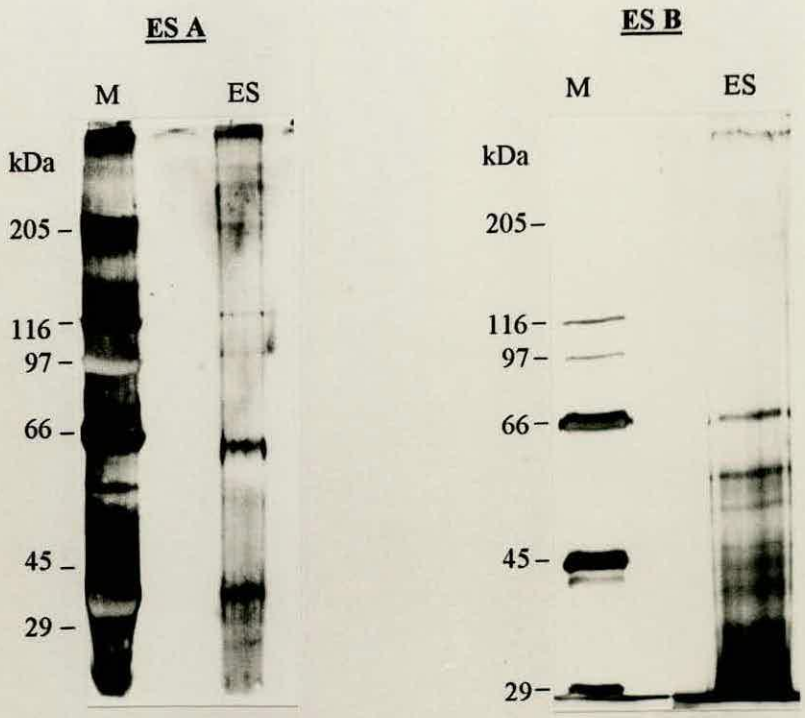
(b) Western blot of adult *T. vitrinus* ES

Adult *T. vitrinus* ES was separated by SDS-PAGE under reducing conditions and Western blotted onto membrane. The blot strips were either non-periodate (NP) or periodate (P) treated and probed with anti-adult *T. vitrinus* ES serum (anti-ES), anti-adult *T. colubriformis* sAChE (anti-sAChE) serum, normal rabbit serum, serum from sheep hyperimmune to *T. vitrinus* infection serum or pre-immune sheep serum. Each antiserum was diluted 1:500.

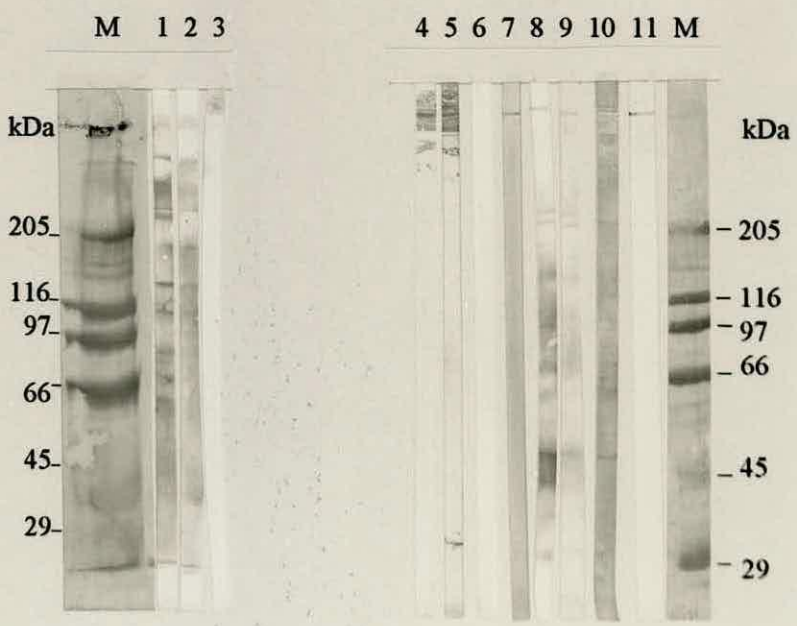
Lanes:

M - high molecular weight protein markers
1 - anti-ES 1; P
2 - anti-ES 1; NP
3 - normal rabbit; NP
4 - anti-sAChE; NP
5 - anti-sAChE; P
6 - normal rabbit; NP
7 - normal rabbit; P
8 - hyperimmune sheep; NP
9 - hyperimmune sheep; P
10 - pre-immune sheep; NP
11 - pre-immune sheep; P

(a)


ES A
M
ES
kDa
205
116
97
66
45
29
ES B
M
ES
kDa
205
116
97
66
45
29

(b)


M 1 2 3
4 5 6 7 8 9 10 11 M
kDa
205
116
97
66
45
29
kDa
205
116
97
66
45
29

5.2.2 Titration of the *T. vitrinus* cDNA λgt11 library

Before immunoscreening the adult *T. vitrinus* cDNA λgt11 library, the titre of the library was measured to determine the dilution required to give the optimal density of plaques for immunoscreening purposes (see section 2.10.10). The results are shown in table 5.1.

The mean titre of the adult *T. vitrinus* cDNA library was 3.5×10^4 pfu/0.1 ml. Using this estimated library titre, *E. coli* cells were infected with $1\text{-}2 \times 10^2$ phage in 100 µl of SM buffer (as described in section 2.10.12) and subsequently plated out onto LB-agar/ampicillin plates (80 mm diameter).

Table 5.1

Titration of adult *T. vitrinus* cDNA λgt11 library.

dilution of adult *T. vitrinus* cDNA λgt11 library	number of plaques formed/0.1 ml	pfu/0.1 ml
10^2	>1000	not determined
10^3	251	2.51×10^4
10^4	51	5.1×10^4
10^5	3	3×10^4
10^6	no plaques formed	-
10^7	no plaques formed	-

5.2.3 Immunoscreening of adult *T. vitrinus* cDNA λgt11 library with anti-adult *T. vitrinus* ES serum

From the adult *T. vitrinus* cDNA λgt11 library approximately 3 x 10^5 phage plaques were screened with anti-adult *T. vitrinus* ES serum (see section 2.10.12) and 34 possible immunopositives were selected based on their reactivity with the antiserum. Figure 5.2 (a) demonstrates the signal given by a strong immunopositive plaque. Weaker immunopositive plaques appeared only fractionally darker than the background negative plaques. The selected plaques were picked, replated and their reactivity with the antiserum confirmed by rescreening. Following this, 14 definite immunopositives remained (KM 1, 2, 8, 12, 13, 16, 18, 19, 22, 26, 30, 31, 33 and 34). The positive plaques were selected, replated and rescreened until plaque purity had been obtained for each. A dot array of the immunopositive plaques screened with the anti-ES serum is shown in figure 5.2 (b). In this particular filter, no KM16 plaques developed.

Following immunoscreening of the clones with normal rabbit serum, KM18 was strongly recognised suggesting that the recombinant protein expressed by this clone was not recognised specifically by the anti-adult *T. vitrinus* ES serum (results not shown). None of the other clones were recognised by the normal serum. The clones were also screened with serum from a sheep hyperimmune to *T. vitrinus*. Again, KM18 reacted with both the immune serum and normal sheep serum. None of the other clones unequivocally reacted with the either sera (results not shown).

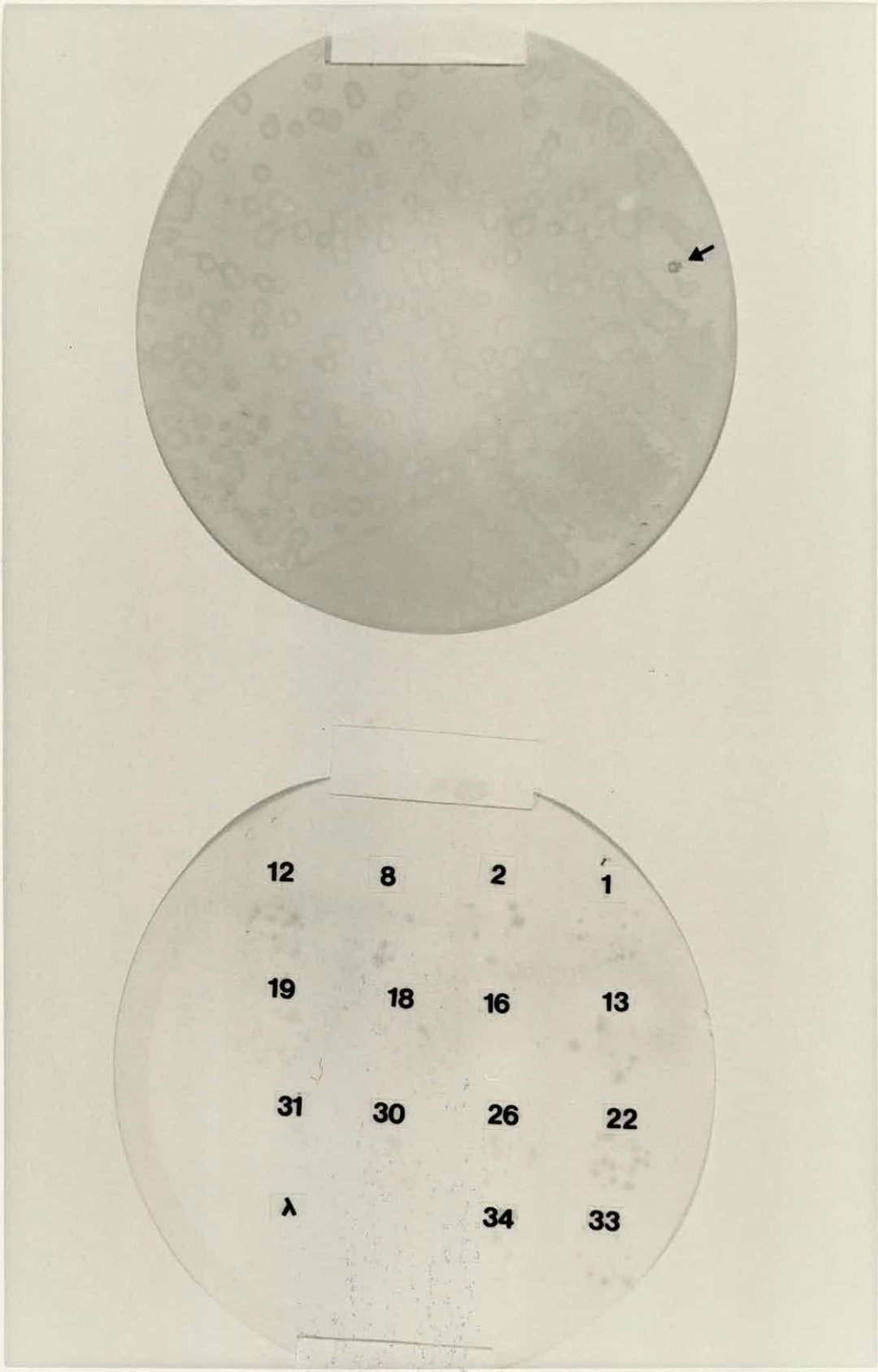
Figure 5.2

(a) Isolation of primary immunopositive phage plaque from a filter of phage immunoscreened with anti-adult *T. vitrinus* ES serum.

An immunopositive clone appeared as a plaque darker in colour to the background immmunonegative plaques.

(b) Dot array of the selected immunopositive plaques

The selected KM immunopositives (1, 2, 8, 12, 13, 16, 18, 19, , 22, 26, 30, 31) and a non-recombinant phage (λ) were spotted onto a lawn of Y1090 cells and overlaid with a nylon filter previously saturated with IPTG. Each phage preparation produced 15-20 plaques, apart from KM16 which produced no plaques. The filter was probed with anti-adult *T. vitrinus* ES serum.


12
8
2
1
19
18
16
13
31
30
26
22
λ
34
33

5.2.4 Analysis of the insert cDNA from immunopositive λgt11 clones

The sizes of the insert cDNA carried in the immunopositive λgt11 clones was determined using PCR and oligonucleotide primers G0507 and G0508 (see table 2.1), which allow amplification of the DNA inserted within λgt11 *Eco*R1 cloning site. KM2 and KM12 were found to contain inserts of < 50 bp in size. The resultant amplifications of the other clones were analysed by 0.8% (w/v) agarose gel electrophoresis (figure 5.3). The insert sizes, excluding the 340 nucleotides of λgt11 arms, were estimated using linear regression by reference to the DNA markers and are listed in table 5.2. The insert sizes range from approximately 1,327 bp (KM1) to 225 bp (KM16 and KM34). Immunopositive KM31 (not shown) gave a DNA insert of variable size following PCR which was indicative of non-specific amplification.

Figure 5.3

Agarose gel analysis of recombinant inserts from immunopositive clones.

The inserts present in the recombinant clones were amplified using PCR and the λgt11 primers, G0507 and G0508. The products from the PCR reactions were electrophoresed through an agarose (0.8%, w/v) gel. The amplified inserts include 340 bp of λgt11.

Lanes:

M - DNA markers
1 - immunopositive KM1
2 - immunopositive KM8
3 - immunopositive KM13
4 - immunopositive KM16
5 - immunopositive KM19
6 - immunopositive KM 22
7 - immunopositive KM26
8 - immunopositive KM30
9 - immunopositive KM 33
10 - immunopositive KM 34

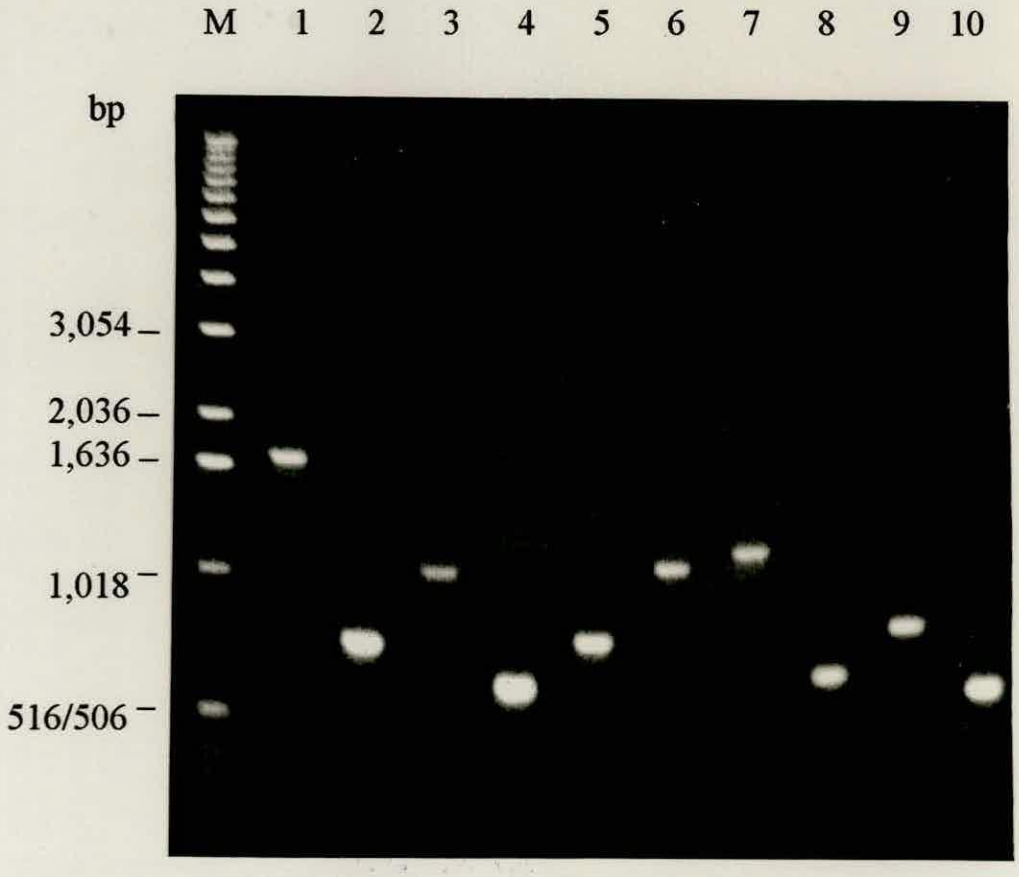

M 1 2 3 4 5 6 7 8 9 10
bp
3,054
2,036
1,636
1,018
516/506

Table 5.2

Size of immunopositive clones recombinant insert DNA

immunopositive clone	approximate size of insert excluding 340 bp λgt11 arms (bp)
KM1	1327
KM8	345
KM13	617
KM16	225
KM19	345
KM22	630
KM26	729
KM30	253
KM33	414
KM34	225

5.2.5 Subcloning and sequencing of the DNA insert from immunopositive KM1

As immunopositive KM1 contained the largest recombinant DNA fragment, this insert cDNA was chosen initially for further analysis. In order to sequence the fragment, it was subcloned into the sequencing vector, pBluescript II SK+. The DNA insert from KM1 was PCR amplified (section 2.11.7) with the λgt11 primers, 514N and 515N (see table 2.2). The resultant 1.33 kb fragment was electrophoresed through a 0.8% (w/v) agarose gel and purified by the Geneclean procedure, followed by a phenol:chlorofrom extraction and ethanol precipitation (see section 2.11). The purified fragment was then digested with *Eco*R1 to remove the λgt11 arms and regenerate the original *Eco*R1 sites at the end of the cDNA that were utilised for cloning into λgt11 (section 2.11.13). *Eco*R1 digestion of the fragment resulted in the production of a smaller fragment of approximately 700 bp (figure 5.4). This suggested that the DNA insert of KM1 contained an internal *Eco*R1 site and was thus cleaved in half. Subsequent DNA-PAGE analysis of the restriction digest confirmed the presence of two fragments of approximately 700 and 720 bp in size (not shown). The restricted fragments were ligated into *Eco*R1 cut pBluescript II SK+, as detailed in section 2.10.7. Insert analysis by DNA-PAGE of the plasmid DNA from transformed cells concluded that only the smaller 700 bp fragment had been successfully subcloned into the sequencing vector (not shown). This fragment was subsequently sequenced by the di-deoxy chain termination method using the M13 forward and reverse primers (section 2.11.15). The fragment was sequenced three times in both directions. The derived nucleotide sequence and the corresponding amino acid sequence are shown in figure 5.5.

The recombinant cDNA is inserted into the λgt11 genome so as to be expressed as a fusion protein with β-galactosidase, therefore, the orientation of the nucleotide sequence is known. The sequenced fragment was 608 bp in total and encoded a peptide of 203 amino acids. Analysis of the peptide sequence using the FastA computer programme concluded that the protein showed significant homology to several types of myosin heavy chain. The best match was to the myosin heavy chain from chicken gizzard smooth muscle (Yanagisawa *et al.*, 1987), with 36.0% identity and 58.2% conservation over 207 amino acids (figure 5.6).

Figure 5.4

Agarose gel analysis of *Eco*R1 digested and uncut 1.33 kb insert from KM1.

The amplified 1.33 kb insert from KM1 was purified and digested with *Eco*R1. The resultant digested DNA was analysed by agarose (0.8%, w/v) gel electrophoresis.

Lanes:

M - DNA markers
1 - KM1 1.33 kb insert digested with *Eco*R1
2 - KM1 1.33 kb insert uncut

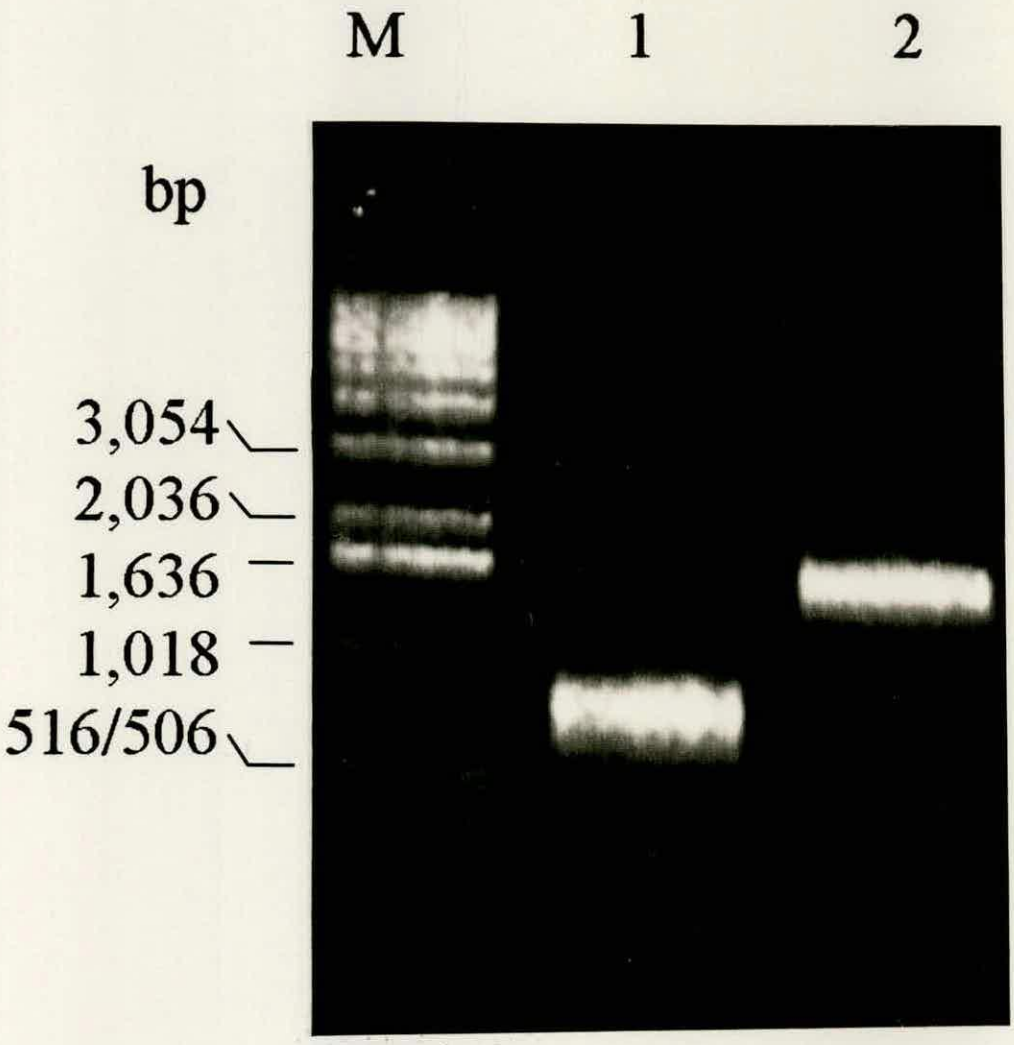

M
1
2
bp
3,054
2,036
1,636
1,018
516/506

Figure 5.5

Nucleotide and derived amino acid sequence of the fragment from KM1.

```
    GTGCAAGCGCTCGAACAGCTTGACAAAGCTCGTGAAGAAATTGATCAGCTGAGTCGTACC
  1 ---------+---------+---------+---------+---------+---------+ 60
    CACGTTCGCGAGCTTGTCGAACTGTTTCGAGCACTTCTTTAACTAGTCGACTCAGCATGG

  1 V  Q  A  L  E  Q  L  D  K  A  R  E  E  I  D  Q  L  S  R  T  20


    CGTGAAGACGAAGAGCAGCTGGTAACGAATTTGAATAGGAAAATTGCTACTCTTGAAGAG
 61 ---------+---------+---------+---------+---------+---------+ 120
    GCACTTCTGCTTCTCGTCGACCATTGCTTAAACTTATCCTTTTAACGATGAGAACTTCTC

 21 R  E  D  E  E  Q  L  V  T  N  L  N  R  K  I  A  T  L  E  E  40


    CAGTTGCATGAGATCAGCGATCAAGTACAGGAAGAGACAAGAGCTAAACTCGCTCAAATT
121 ---------+---------+---------+---------+---------+---------+ 180
    GTCAACGTACTCTAGTCGCTAGTTCATGTCCTTCTCTGTTCTCGATTTGAGCGAGTTTAA

 41 Q  L  H  E  I  S  D  Q  V  Q  E  E  T  R  A  K  L  A  Q  I  60


    AATCGTGTTCGGCAACTTGAAGAAGAGAAAGCAGCCCTAGTTGAAGATAGGGACGAGCTG
181 ---------+---------+---------+---------+---------+---------+ 240
    TTAGCACAAGCCGTTGAACTTCTTCTCTTTCGTCGGGATCAACTTCTATCCCTGCTCGAC

 61 N  R  V  R  Q  L  E  E  E  K  A  A  L  V  E  D  R  D  E  L  80


    GACGCCTGCACGTCAACATATGGAACGGACATCATGATGGTGCGCCAGCTTGTTGATCGA
241 ---------+---------+---------+---------+---------+---------+ 300
    CTGCGGACGTGCAGTTGTATACCTTGCCTGTAGTACTACCACGCGGTCGAACAACTAGCT

 81 D  A  C  T  S  T  Y  G  T  D  I  M  M  V  R  Q  L  V  D  R  100


    AGAAGCGATGAGGGTTATACAACAATGGAGGAGGCTGAGAAGAAGGCTCAACGCGATTTG
301 ---------+---------+---------+---------+---------+---------+ 360
    TCTTCGCTACTCCCAATATGTTGTTACCTCCTCCGACTCTTCTTCCGAGTTGCGCTAAAC

101 R  S  D  E  G  Y  T  T  M  E  E  A  E  K  K  A  Q  R  D  L  120


    GAGAACTGCCAACATATGCTTGAGGAAAGCGAGGCTGGAAAGGAACGTCTCATCCAAAGC
361 ---------+---------+---------+---------+---------+---------+ 420
    CTCTTGACGGTTGTATACGAACTCCTTTCGCTCCGACCTTTCCTTGCAGAGTAGGTTTCG

121 E  N  C  Q  H  M  L  E  E  S  E  A  G  K  E  R  L  I  Q  S  140
```

```
    AAGAAGAAGCTTCAACAAGAGCTGGAAGACGCCAACATTGAATTGGAGAACATTCGTACG
421 ---------+---------+---------+---------+---------+---------+ 480
    TTCTTCTTCGAAGTTGTTCTCGACCTTCTGCGGTTGTAACTTAACCTCTTGTAAGCATGC

141 K  K  K  L  Q  Q  E  L  E  D  A  N  I  E  L  E  N  I  R  T  160


    GCTTCTAGGGAAATGGAGAAGCGACAGAAGAAGTTTGACATGCAGCTGGCTGAGGAGAGA
481 ---------+---------+---------+---------+---------+---------+ 540
    CGAAGATCCCTTTACCTCTTCGCTGTCTTCTTCAAACTGTACGTCGACCGACTCCTCTCT

161 A  S  R  E  M  E  K  R  Q  K  K  F  D  M  Q  L  A  E  E  R  180


    GCCAACGTGCAAAAGGCTATCCTTGAACGAGACGCCCACGCACAAGAGTCACGCGATCGA
541 ---------+---------+---------+---------+---------+---------+ 600
    CGGTTGCACGTTTTCCGATAGGAACTTGCTCTGCGGGTGCGTGTTCTCAGTGCGCTAGCT

181 A  N  V  Q  K  A  I  L  E  R  D  A  H  A  Q  E  S  R  D  R  200


    GAAACACG
601 -------- 608
    CTTTGTGC

201 E  T  R  203
```

The sequenced fragment from KM1 (which contained an insert of 1.33 kb in total) was 608 bp in length and encoded a peptide of 203 amino acids. The upper nucleotide sequence corresponds to the sense strand and reads 5' to 3'. The lower nucleotide sequence is the antisense strand and reads 3' to 5'. The derived amino acid sequence is written in bold print and each amino acid is written directly underneath the first nucleotide of the codon. The first and last nucleotide or amino acid in each line is numbered.

Figure 5.6

Alignment of the deduced amino acid sequence for KM1 insert with a homologuous segment from chicken gizzard smooth muscle myosin heavy chain.

```
KM1 X Q A L E Q L D K A R E E I D Q L S R T R E D E E 25
MYO T E L N E K V H K L Q I E V E N V T S L L N E A E 1305

KM1 Q L V T N L N R K I A T L E E Q L H E I S D Q V Q 50
MYO S K N I K L T K D V A T L G S Q L Q D T Q E L L Q 1330

KM1 E E T R A K L A Q I N R V R Q L E E E K A A L V E 75
MYO E E T R Q K L N V T T K L R Q L E D D K N S L Q E 1355

KM1 D R D E L D A C T S T Y G T D I - M M V R Q L V D 99
MYO Q L D E E V E A K Q N L E R H I S T L T I Q L S D 1380

KM1 R R S D - E G Y T - - - - T M E E A E K K A Q R D 119
MYO S K K K L Q E F T A T V E T M E E G K K K L Q R E 1405

KM1 L E N C Q H M L E E S E A G K E R L I Q S K K K L 144
MYO I E S L T Q Q F E E K A A S Y D K L E K T K N R L 1430

KM1 Q Q E L E D A N I E L E N I R T A S R E M E K R Q 179
MYO Q Q E L D D L V V D L D N Q R Q L V S N L E K K Q 1455

KM1 K K F D M Q L A E E R A N V Q K A I L E R D A H A 194
MYO K K F D Q M L A E E K N I S S K Y A D E R D R A E 1480

KM1 Q E S R D R E T 202
MYO A E A R E K E T 1488
```

The deduced amino acid sequence from the KM1 insert was aligned with a similar section of chicken myosin heavy chain (MYO; Yanagisawa *et al.*, 1987). Over 207 amino acids there was 36.0% identity (▒) and 58.2% conservation (▒). Gaps in the KM1 sequence are represented by dashes (-). The numbers correspond to the position of the adjacent amino acids in the peptide sequence.

5.2.6 Sequencing and analysis of the insert cDNA from other immunopositive λgt11 clones

The DNA inserts from the remaining clones were amplified by PCR using the oligonucleotide primers G0507 and G0508 (see table 2.2). The resultant fragments were electrophoresed through a 0.8% (w/v) agarose gel and were purified by the Geneclean technique, followed by a phenol:chloroform extraction and ethanol precipitation (section 2.11). The inserts were then directly sequenced using the primer 514N, by Ms. J. Bartley, University of Durham. UK. The sequences for KM2 and KM12 were not obtained as the insert sizes were very small. The nucleotide sequences from a single sequence reading obtained for KM8, 13, 16, 22, 26, 30, 33 and 34 are shown in figure 5.7. The complete insert sequence was attained for clones KM8, 16 and 30.

As each insert had only been sequenced once, each nucleotide sequence was translated into all three possible open reading frames to allow for possible errors in the sequences. Upon analysis, none of the translated peptides for KM8, 13, 16, 22, 26, 30 and 34 showed significant homology to any of the sequences currently in the computer databases. For KM33, 330 bp of the insert (total size approximately 550 bp) was sequenced and encoded a peptide of 110 amino acids (figure 5.7 (g)). This peptide showed 42% identity and 57% conservation over 118 amino acids with the *C. elegans* vitellogenin-6 precursor (Spieth and Blumenthal, 1985; figure 5.8). The KM33 peptide also was >30% identical to *C. elegans* vitellogenin-5 (Spieth *et al.*, 1985) and vitellogenin-4 (Spieth *et al.*, 1985).

For KM19, the insert was sequenced as described above for the other clones. On analysis of the peptides translated from the three possible open reading frames, none of them matched any of the sequences currently on the computer databases. However, translation of the KM19 nucleotide sequence in the three open reading frames opposite to the orientation of β-galactosidase identified a peptide that was significantly homologous to several types of serine proteinase inhibitor (serpin). The sequence analysis of the insert cDNA from immunopositive KM19 is discussed in detail in chapter six.

Figure 5.7

Partial nucleotide sequence of the cDNA recombinant inserts from immunopositives (a) KM8, (b) KM13, (c) KM16, (d) KM22, (e) KM26, (f) KM30, (g) KM33 and (h) KM34.

A single sequence read from the 5' end of each clone (using primer 514N) was obtained. For each sequence, both the sense (top sequence, reading 5' to 3') and the antisense (bottom sequence, reading 3' to 5') strands are shown. In the sequences 'N' denotes the nucleotides A, C, G or T. The amount of nucleotide sequence obtained for each clone was as follows:

KM8 - 369 bp
KM13 - 120 bp
KM16 - 241 bp
KM22 - 204 bp
KM26 - 191 bp
KM30 - 235 bp
KM33 - 330 bp
KM34 - 219 bp

For KM33, the derived amino acid sequence is also shown in bold and each amino acid is written directly beneath the first nucleotide in the codon.

(a) Clone KM8

```
    GCAGTAATCCACGCCCTGGCTCTCACTGACTTTTATACTTTGTGACAAGCTGTTACAACG
  1 ---------+---------+---------+---------+---------+---------+ 60
    CGTCATTAGGTGCGGGACCGAGAGTGACTGAAAATATGAAACACTGTTCGACAATGTTGC

    ACATCCTCCTCGGAAATAGATTGAGCCCAATTCGGTAAGCGCTATGATCTCTGTTCTCTA
 61 ---------+---------+---------+---------+---------+---------+ 120
    TGTAGGAGGAGCCTTTATCTAACTCGGGTTAAGCCATTCGCGATACTAGAGACAAGAGAT

    ATCATAGCGTTAGAGAACTCCAGTAGCGCTCCAAATGGACTACGTGTATTGATCGTGCCG
121 ---------+---------+---------+---------+---------+---------+ 180
    TAGTATCGCAATCTCTTGAGGTCATCGCGAGGTTTACCTGATGCACATAACTAGCACGGC

    TATATGCACACTCGGAGAGAGTGAAATGGGAGAGCAGNGGTTCAAAAGCTGTNTGNCAGA
181 ---------+---------+---------+---------+---------+---------+ 240
    ATATACGTGTGAGCCTCTCTCACTTTACCCTCTCGTCNCCAAGTTTTCGACANACNGTCT

    GGAATACTATGAGCAATTTATCAGGGAAGGCGTTTATCCTGATGGACGTTCGGTATTAGC
241 ---------+---------+---------+---------+---------+---------+ 300
    CCTTATGATACTCGTTAAATAGTCCCTTCCGCAAATAGGACTACCTGCAAGCCATAATCG

    GTTTAATTCGCTATTTTTCAAGTGTGGTGCTTGGNGAGGTGTTGGATCCGCTTTGGGTAA
301 ---------+---------+---------+---------+---------+---------+ 360
    CAAATTAAGCGATAAAAAGTTCACACCACGAACCNCTCCACAACCTAGGCGAAACCCATT

    AACTGGGAG
361 --------- 369
    TTGACCCTC
```

(b) Clone KM13

```
    GCTTTCCACTGGTCCCTGAGACCGTTCCTGAAGAGAAGAAAGCTCAGGGCTGAGATTAGA
  1 ---------+---------+---------+---------+---------+---------+ 60
    CGAAAGGTGACCAGGGACTCTGGCAAGGACTTCTCTTCTTTCGAGTCCCGACTCTAATCT

    GCAAAGACCCTGCAGAACAAGGTCAAGCTTGCCGCTATGAACAAGGAGAAGANGGCGACG
 61 ---------+---------+---------+---------+---------+---------+ 120
    CGTTTCTGGGACGTCTTGTTCCAGTTCGAACGGCGATACTTGTTCCTCTTCTNCCGCTGC
```

(c) Clone KM16

```
    GGGGGAGGATAAAAGTAAACTGGCATGAACAAAACCATGGCAAGTCACCTTTTCCACAAT
  1 ---------+---------+---------+---------+---------+---------+ 60
    CCCCCTCCTATTTTCATTTGACCGTACTTGTTTTGGTACCGTTCAGTGGAAAAGGTGTTA

    ATCTATCAAGTCGCGGAAACATTTCAATAATGAATTTGCTACCTTGAATATCGTTAGTAT
 61 ---------+---------+---------+---------+---------+---------+ 120
    TAGATAGTTCAGCGCCTTTGTAAAGTTATTACTTAAACGATGGAACTTATAGGAATCATA

    TGTTAAATAGGAAATACGATNCCGCTAAAAAAAAGATTGTTATGCTCGTAAACAACAAAG
121 ---------+---------+---------+---------+---------+---------+ 180
    ACAATTTATCCTTTATGCTANGGCGATTTTTTTTCTAACAATACGAGCATTTGTTGTTTC

    TCTAGTACAATGTTTGATANGCAAATNGCTCAGCGACAATGGGAAATATGAAACTCGGGG
181 ---------+---------+---------+---------+---------+---------+ 240
    AGATCATGTTACAAACTATNCGTTTANCGAGTCGCTGTTACCCTTTATACTTTGAGCCCC

    C
241 - 241
    G
```

(d) Clone KM22

```
    GTGATAGTGAATGCTGGCCGTGAACTGTCCCTTCTGATGTATCATGTTCTGCTCGAGCCG
  1 ---------+---------+---------+---------+---------+---------+ 60
    CACTATCACTTACGACCGGCACTTGACAGGGAAGACTACATAGTACAAGACGAGCTCGGC

    ATATTGGACTACCTCTATCAGCGGTATAAAATCATCGAAACGGTGATTCTTATCTATTTC
 61 ---------+---------+---------+---------+---------+---------+ 120
    TATAACCTGATGGAGATAGTCGCCATATTTTAGTAGCTTTGCCACTAAGAATAGATAAAG

    CTTGGTCCTGTATGCGATACAATCGTGAAGAACATACCGGAGAAGAGTCCATTCTGTGAT
121 ---------+---------+---------+---------+---------+---------+ 180
    GAACCAGGACATACGCTATGTTAGCACTTCTTGTATGGCCTCTTCTCAGGTAAGACACTA

    GACAGCGATGTCGAACTGGAAGGT
181 ---------+---------+---- 204
    CTGTCGCTACAGCTTGACCTTCCA
```

(e) Clone KM26

```
    ATTGTAATCGCGGAGCGCGCGAGGTATCTCTTTTGCATTTTCCAGCCGCTTCGCAGGAAA
  1 ---------+---------+---------+---------+---------+---------+ 60
    TAACATTAGCGCCTCGCGCGCTCCATAGAGAAAACGTAAAAGGTCGGCGAAGCGTCCTTT

    AACGAATCCTNCCGGATACTGAGGATCTTCATAGATCACCATCATTGTTCCATTTACAGG
 61 ---------+---------+---------+---------+---------+---------+ 120
    TTGCTTAGGANGGCCTATGACTCCTAGAAGTATCTAGTGGTAGTAACAAGGTAAATGTCC

    CAAATCAGGGCACGACTCGTCGTNGACAGGAGATGGAAATGGCAGATCGGGAGCGAGAGC
121 ---------+---------+---------+---------+---------+---------+ 180
    GTTTAGTCCCGTGCTGAGCAGCANCTGTCCTCTACCTTTACCGTCTAGCCCTCGCTCTCG

    CGTTTCGTCAG
181 ---------+- 191
    GCAAAGCAGTC
```

(f) Clone KM30

```
    GGGGCGTTATTGACGTCATAGGCTGGCTTTCCTACTGGAATATCCCTGTTGCACTTCTTA
  1 ---------+---------+---------+---------+---------+---------+ 60
    CCCCGCAATAACTGCAGTATCCGACCGAAAGGATGACCTTATAGGGACAACGTGAAGAAT

    CAGTTCGCGGGTCTCGATGNGGTTTTCGGAACGGGACTATCTCCCTTACAGNAGTCTTTG
 61 ---------+---------+---------+---------+---------+---------+ 120
    GTCAAGCGCCCAGAGCTACNCCAAAAGCCTTGCCCTGATAGAGGGAATGTCNTCAGAAAC

    CACCTAGGCATGTGATCAATTTCATAAAATGGCGTTCCAGGAGAGAGTTCTTTCCAGCAT
121 ---------+---------+---------+---------+---------+---------+ 180
    GTGGATCCGTACACTAGTTAAAGTATTTTACCGCAAGGTCCTCTCTCAAGAAAGGTCGTA

    CTCCTGCATTGNACCGGCTTCGGNGAAGNTTGTAGAGGTGGTTTGGTACCATCAG
181 ---------+---------+---------+---------+---------+----- 235
    GAGGACGTAACNTGGCCGAAGCCNCTTCNAACATCTCCACCAAACCATGGTAGTC
```

(g) Clone KM33

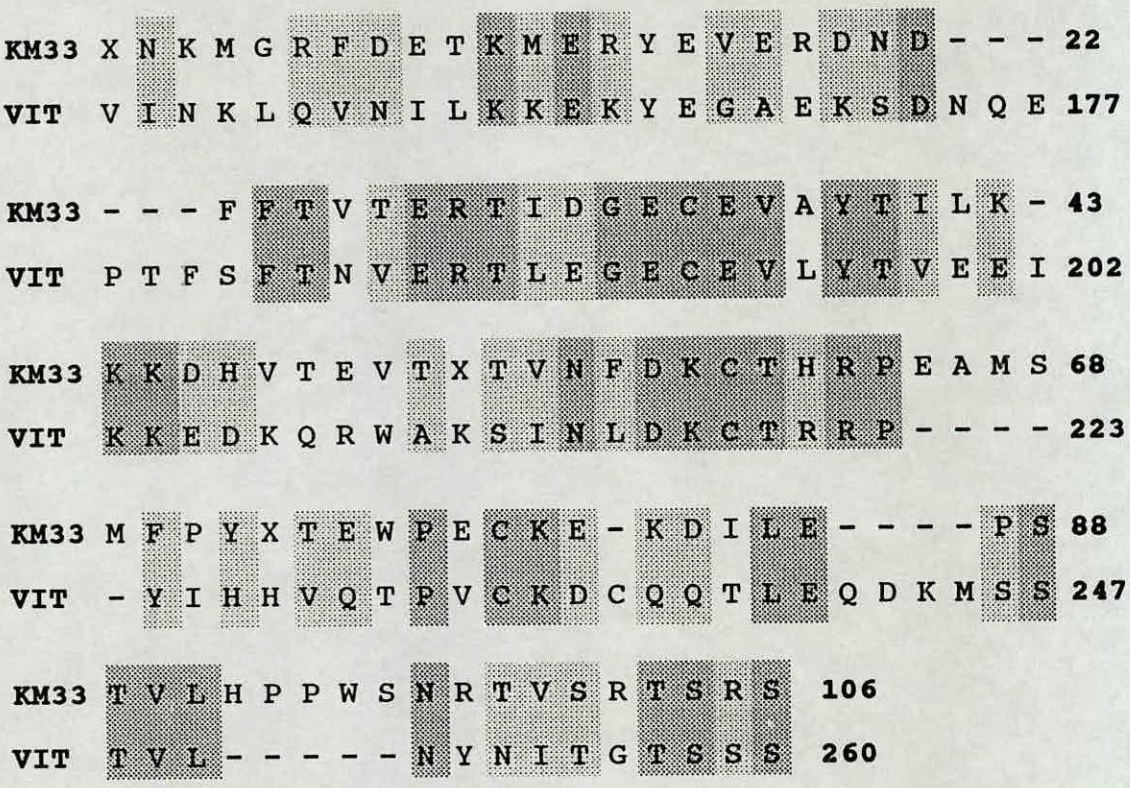
```
    GTGAACAAGATGGGACGATTTGACGAGACCAAAATGGAGAGATACGAAGTAGAACGTGAC
  1 ---------+---------+---------+---------+---------+---------+ 60
    CACTTGTTCTACCCTGCTAAACTGCTCTGGTTTTACCTCTCTATGCTTCATCTTGCACTG

    X  N  K  M  G  R  F  D  E  T  K  M  E  R  Y  E  V  E  R  D   20


    AACGACTTCTTCACCGTTACCGAGAGAACCATCGATGGAGAGTGCGAGGTTGCCTACACT
 61 ---------+---------+---------+---------+---------+---------+ 120
    TTGCTGAAGAAGTGGCAATGGCTCTCTTGGTAGCTACCTCTCACGCTCCAACGGATGTGA

    N  D  F  F  T  V  T  E  R  T  I  D  G  E  C  E  V  A  Y  T   40


    ATCCTCAAGAAGAAGGACCACGTCACTGAGGTTACCANGACCGTCAACTTCGACAAGTGC
121 ---------+---------+---------+---------+---------+---------+ 180
    TAGGAGTTCTTCTTCCTGGTGCAGTGACTCCAATGGTNCTGGCAGTTGAAGCTGTTCACG

    I  L  K  K  K  D  H  V  T  E  V  T  X  T  V  N  F  D  K  C   60


    ACTCATCGCCCAGAGGCCATGTCCATGTTCCCCTATNTCACTGAGTGGCCCGAATGCAAG
181 ---------+---------+---------+---------+---------+---------+ 240
    TGAGTAGCGGGTCTCCGGTACAGGTACAAGGGGATANAGTGACTCACCGGGCTTACGTTC

    T  H  R  P  E  A  M  S  M  F  P  Y  X  T  E  W  P  E  C  K   80


    GAGAAGGATATACTGGAGCCAAGTACTGTTCTACACCCACCCTGGTCGAATAGGACGGTC
241 ---------+---------+---------+---------+---------+---------+ 300
    CTCTTCCTATATGACCTCGGTTCATGACAAGATGTGGGTGGGACCAGCTTATCCTGCCAG

    E  K  D  I  L  E  P  S  T  V  L  H  P  P  W  S  N  R  T  V   100


    TCAAGAACGTCGAGGTCCCTTCCATGTCTT
301 ---------+---------+---------+ 330
    AGTTCTTGCAGCTCCAGGGAAGGTACAGAA

    S  R  T  S  R  S  L  P  C  L   110
```

(h) Clone KM34

```
    GATCATTCACATTTGTGCATCTTTCATCAGATGGTAATTTTGAACACTAATAAATTATTA
  1 ---------+---------+---------+---------+---------+---------+ 60
    CTAGTAAGTGTAAACACGTAGAAAGTAGTCTACCATTAAAACTTGTGATTATTTAATAAT

    CTCTGCAAAAAAAAAAAAAAAANNGGGGNNNCGGGGGGGAAAGGNTNCCCAANGGTNNAA
 61 ---------+---------+---------+---------+---------+---------+ 120
    GAGACGTTTTTTTTTTTTTTTNNCCCCNNNGCCCCCCCTTTCCNANGGGTTNCCANNTT

    ANTNNTTTNGGGGNNAATTNNNNTTGGGGGGAAAANNAANGTTTTAATNGGGGGGNCNNN
121 ---------+---------+---------+---------+---------+---------+ 180
    TNANNAAANCCCCNNTTAANNNNAACCCCCCTTTTNNTTNCAAAATTANCCCCCCNGNNN

    NANGGNGNAANGGGGGGGNGGGNGGNNCCCTGGGGGGGGG
181 ---------+---------+---------+--------- 219
    NTNCCNCNTTNCCCCCCCNCCCNCCNNGGGACCCCCCCCC
```

Figure 5.8

Alignment of the deduced amino acid sequence for KM33 with a homologous segment of *C. elegans* vitellogenin-6 precursor.



The deduced amino acid sequence from the nucleotide sequence of the KM33 insert was aligned to a similar region of *C. elegans* vitellogenin-6 precursor (VIT; Spieth and Blumenthal, 1985). Over 118 amino acids there was 42.0% identity (▒) and 57.0% conservation (▒). A dash (-) in the amino acid sequence represents a gap. The numbers correspond to the position of the adjacent amino acids in the peptide sequence.

5.2.7 Analysis of recombinant lysogens of immunopositive clones

Recombinant lysogens were prepared from a selection of the immunopositive clones: KM1, 8, 13, 16, 19, 26 and 30 (see section 2.10.14). The expressed β-galactosidase fusion proteins were analysed by reducing SDS-PAGE (7.5%, w/v, polyacrylamide) and Western blotting. Induction was carried out initially, for 1 hr. Induction of *E. coli* cells harbouring non-recombinant λgt11 results in the expression of β-galactosidase, which is 116 kDa in size. In recombinant phage, the β-galactosidase fusion protein would be expected to be larger dependent on the size of the inserted cDNA. Given that the average weight of an amino acid is 126.7 Da (Sambrook, Fritsch and Maniatis, 1989), the expected size of the β-galactosidase fusion proteins from KM1, 8, 13, 16, 19, 26 and 30 were approximately 175, 131, 145, 135, 132, 148 and 128 kDa, respectively.

The expression of a 116 kDa protein was clearly induced in bacteria infected with non-recombinant λgt11 (figure 5.9, lane 16). Reducing SDS-PAGE and Coomassie staining analysis demonstrated that fusion proteins of approximately the correct predicted size were observed following induction of KM1 (180 kDa, figure 5.9, lane 2) and KM26 (148 kDa, figure 5.9, lane 13) as these proteins were not evident in the corresponding uninduced samples (figure 5.9, lane 1 and lane 14). A protein doublet at 116 kDa was faintly evident in induced KM30 (figure 5.9, lane 12) which was not evident in the uninduced sample (figure 5.9, lane 11) despite the comparable protein loading. Induction resulted in the increased expression of proteins around 116 kDa in KM8, 13 and 16 (figure 5.9, lanes 4, 6 and 8 respectively) while no additional proteins were evident upon induction of KM19 (figure 5.9, lane 10 compared to lane 11).

Fusion protein expression was also examined by Western blot analysis using rabbit anti-*T. vitrinus* ES serum. However, positive signals were only clearly observed with KM1 and KM19 (not shown). The analysis was repeated for KM1 and KM19 as well as non-recombinant λgt11 (figure 5.10) using different induction times for KM19 (figure 5.10 (a) and (b); KM19, lanes 3-8) probed with rabbit anti-*T. vitrinus* ES serum (a) and normal rabbit serum (b). The 180 kDa protein evident in KM1 was recognised by both sera although recognition of this protein as well as a

protein at about 150 kDa was much stronger with the anti-ES serum (compare lane 2, (a) and lane 2 (b)). With induced KM19 as antigen, several peptides around 100 to 120 kDa were recognised by the anti-ES antiserum (figure 5.10 (a), lanes 4-8) but not by the normal rabbit serum (figure 5.10 (b), lanes 4-8) and the strength of the signal appeared to increase with time of induction (compare lane 8 with lane 4, figure 5.10 (a)).

Fusion proteins of the expected size were not demonstrated for the remaining recombinants tested. Attempts to optimise expression by altering the induction period did not enhance expression (not shown).

Figure 5.9

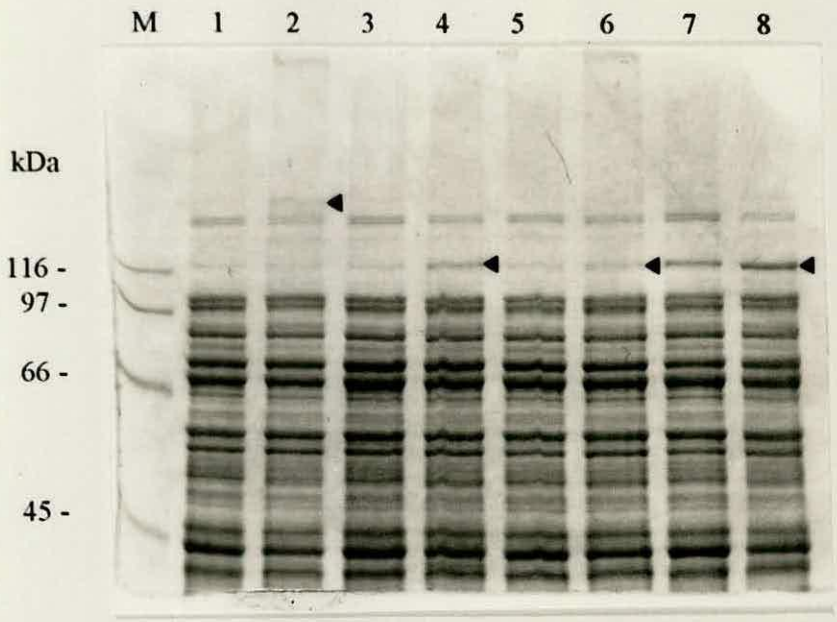
Reducing SDS-PAGE of recombinant lysogens of immunopositive clones.

Recombinant *E. coli* lysogens of KM1, 8, 13, 16, 19, 26 and 30 were prepared with the β-galactosidase fusion protein being induced with IPTG for 1 hr. A lysogen of non-recombinant λgt11 was also prepared. The cell pellets were suspended in reducing SDS-PAGE loading buffer to promote lysis. Induced (I) and uninduced (UI) samples were analysed by 7.5% (w/v) SDS-PAGE and the gels were Coomassie stained for protein. Arrows indicate expressed recombinant proteins.

Lanes:

M - high molecular weight protein markers

1 - immunopositive KM1; UI	2 - immunopositive KM1; I
3 - immunopositive KM8; UI	4 - immunopositive KM8; I
5 - immunopositive KM13; UI	6 - immunopositive KM13; I
7 - immunopositive KM16; UI	8 - immunopositive KM16; I
9 - immunopositive KM19; UI	10 - immunopositive KM19; I
11 - immunopositive KM30; UI	12 - immunopositive KM30; I
13 - immunopositive KM26; I	14 - immunopositive KM26; UI
15 - non-recombinant λgt11;UI	16 - non-recombinant λgt11; I


M 1 2 3 4 5 6 7 8
kDa
116 -
97 -
66 -
45 -

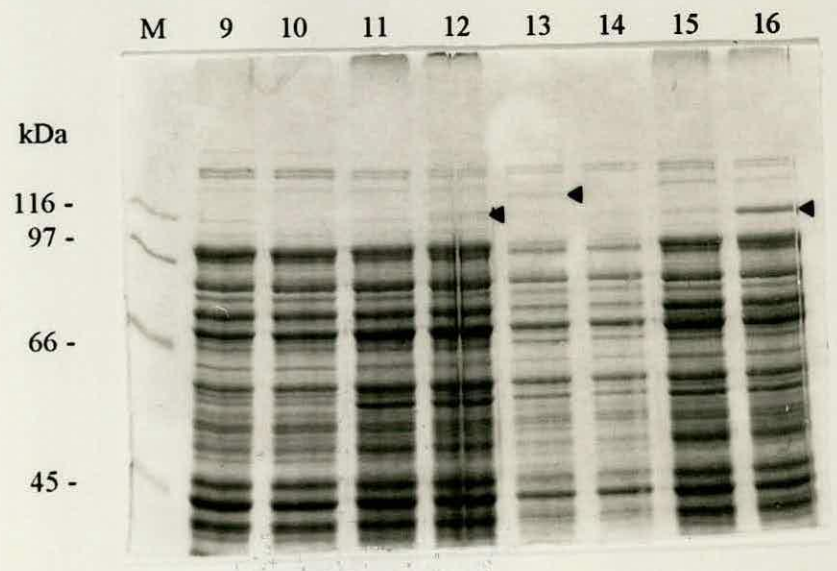

M 9 10 11 12 13 14 15 16
kDa
116 -
97 -
66 -
45 -

Figure 5.10

Western blot analysis of recombinant lysogens of immunopositives KM1 and KM19.

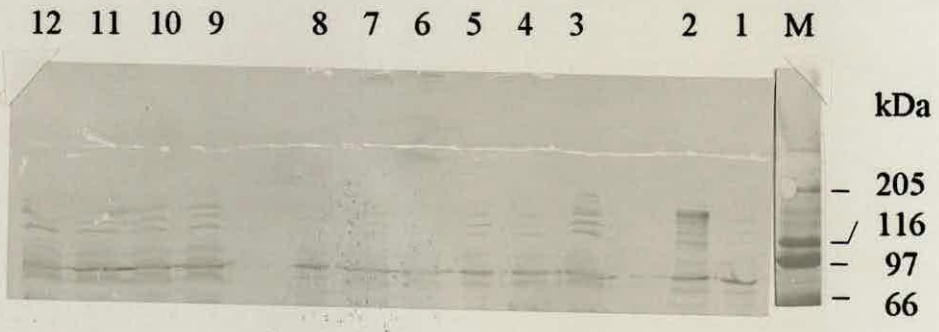
Cell lysates of uninduced (UI) and induced (I) recombinant *E. coli* lysogens of KM1 and KM19 were repeated. KM1 was induced for 60 min, as before. KM19 was induced for 30, 40, 50, 60 or 70 min. Lysogens of non-recombinant λgt11 and uninfected Y1090 cells were also prepared and induced for 60 min. The samples were fractionated by 7.5% (w/v) SDS-PAGE, under reducing conditions, before being Western blotted onto membrane. The blots were probed with either (a) anti-adult *T. vitrinus* ES serum or (b) normal rabbit serum.

Lanes:
M - high molecular weight protein markers
1 - immunopositive KM1; UI
2 - immunopositive KM1; I
3 - immunopositive KM19; UI
4 - immunopositive KM19; I (30 min)
5 - immunopositive KM19; I (40 min)
6 - immunopositive KM19; I (50 min)
7 - immunopositive KM19; I (60 min)
8 - immunopositive KM19; I (70 min)
9 - non-recombinant λgt11; UI
10 - non-recombinant λgt11; I
11 - Y1090 cells; UI
12 - Y1090 cells; I

(a)


12 11 10 9 8 7 6 5 4 3 2 1 M
kDa
205
116
97
66

(b)


12 11 10 9 8 7 6 5 4 3 2 1 M
kDa
205
116
97
66

5.2.8 Immunoscreening of adult *T. vitrinus* cDNA λgt11 library with anti-adult *T. colubriformis* serum

The anti-adult *T. colubriformis* sAChE serum was used to immunoscreen approximately 1600 phage plaques (as described in section 2.10.12) and a single strong immunopositive clone was isolated (designated KM35). The plaque was removed into buffer, replated and rescreened until plaque purity had been obtained.

5.2.9 Analysis of the insert from immunopositive KM35

The size of the recombinant cDNA contained in KM35 was determined as previously described for the other immunopositive clones, using PCR and the λgt11 directed primers 514N and 515N. The amplified fragment was analysed by 0.8% (w/v) agarose gel electrophoresis and was found to be approximately 2,000 bp in size (figure 5.11).

5.2.10 Sequence analysis of KM35

KM35 was amplified with the λgt11 primers G0507 and G0508 (see table 2.2). The fragment was electrophoresed through an agarose gel then purified by Genecleaning (section 2.11.9), followed by phenol:chloroform extraction (section 2.11.1) and precipitated with ethanol (section 2.11.2). The insert was then directly sequenced, using the λgt11 primers 514N/515N and KM35 internal primers G6171/G6172 (deduced from primary sequence data), by Ms. J. Bartley, University of Durham, UK. Assuming that the fragment was in-frame with the λgt11 *lacZ* gene, 680 and 617 bp was sequenced at the 5' and 3' ends of the DNA insert, respectively. The derived nucleotide sequences and the corresponding peptides are shown in figure 5.12 (a) and (b).

The 5' peptide sequence was shown to match several types of heavy chain myosin, with the best homology exhibited by *C. elegans* myosin heavy chain A (Dibb *et al.*, 1989) where 65.6% identity and 74.1% similarity was observed over 226 amino acids (figure 5.13 (a)). The 3' end of KM35 also showed significant homology to the *C. elegans* myosin heavy chain A, with 72.1% identity and 82.8% conservation over 157 amino acids (figure 5.13 (b)).

In the peptide alignment of the 3' end of KM35 with the *C. elegans* myosin, the homology diminished over the last 22 amino acids of the KM35 fragment. On analysis of the nucleotide sequence of the 3' end of KM35, the fragment shared 74.2% homology with the *C. elegans myo*-3 gene for myosin heavy chain 3 (which encodes for myosin heavy chain-A; Dibb *et al.*, 1989) over 453 bp of KM35 3' end (bases 1 to 453; figure 5.14). In the *C. elegans* gene bp 10015 to 10092 carries an intron sequence, which is therefore not represented in the KM35 cDNA sequence. The nucleotide analysis also showed that the complement reverse sequence of this fragment (antisense strand), reading from 576 - 314 bp, was 85.7% identical to 266 bp of the *T. colubriformis* tropomyosin mRNA (Frenkel *et al.*, 1989). The 266 bp in the tropomyosin mRNA corresponds to 5' untranslated sequence found directly before the start codon for the gene.

Figure 5.11

Agarose gel analysis of recombinant insert from immunopositive clone KM35.

The insert present in the recombinant clone KM35 was amplified using PCR and the λgt11 primers, 514N and 515N. The product from the PCR reaction was electrophoresed through an agarose (0.8%, w/v) gel. The amplified inserts include 92 bp of λgt11.

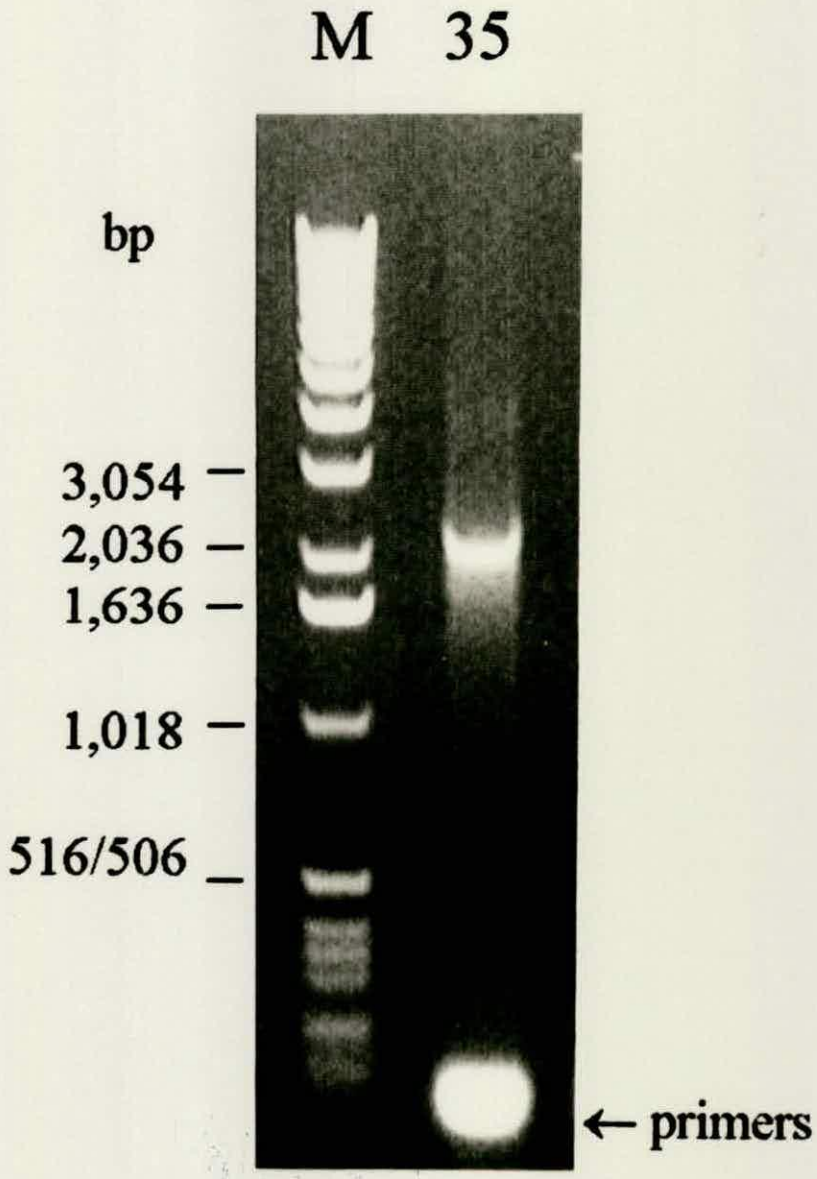

M 35
bp
3,054
2,036
1,636
1,018
516/506
← primers

Figure 5.12

Nucleotide and derived amino acid sequence of the (a) 5' and (b) 3' ends of the insert for immunopositive KM35.

Immunopositive KM 35 was approximately 2,000 bp in size and 680 and 617 bp of the 5' and 3' ends of the insert were sequenced respectively. For each sequence, the upper nucleotide sequence corresponds to the sense strand and reads 5' to 3'. The lower nucleotide sequence is the antisense strand and reads 3' to 5'. The derived amino acid sequence is written in bold and each amino acid is written directly underneath the first nucleotide in the codon. In the nucleotide sequence 'N' denotes A, C, G or T. The first and last nucleotide or amino acid in each line is numbered. The sense (G6172) and antisense (G6171) primers which were designed to the 5' and 3' sequences respectively, are shown.

(a) The nucleotide and derived amino acid sequences for the 5' end of KM35

```
    GTAAACTACGATGAAGAAGCTCGTGAACGCCAAGCCTTGGCTGCCCAGGTCATAACCTTC
  1 ---------+---------+---------+---------+---------+---------+ 60
    CATTTGATGCTACTTCTTCGAGCACTTGCGGTTCGGAACCGACGGGTCCAGTATTGGAAG

  1 V  N  Y  D  E  E  A  R  E  R  Q  A  L  A  A  Q  V  I  T  F  20

    GAGCATGAGAATGACAGCCTTCGTGATCAACTCGCACAGGAATCAGAAGCCAAGGCCGAA
 61 ---------+---------+---------+---------+---------+---------+ 120
    CTCGTACTCTTACTGTCGGAAGCACTAGTTGAGCGTGTCCTTAGTCTTCGGTTCCGGCTT

 21 E  H  E  N  D  S  L  R  D  Q  L  A  Q  E  S  E  A  K  A  E  40

    TTGCTGCGACAAATCAGGAAGCAAAACGCCGAAATCCAACAATGGAAGGCCCGATTTGAA
121 ---------+---------+---------+---------+---------+---------+ 180
    AACGACGCTGTTTAGTCCTTCGTTTTGCGGCTTTAGGTTGTTACCTTCCGGGCTAAACTT

 41 L  L  R  Q  I  R  K  Q  N  A  E  I  Q  Q  W  K  A  R  F  E  60

    AGCGAAGGTCTCGCCAAGCTCGATGAAATCGAGGAGGCAAAGCGCAAGCTGCAAGGCAAG
181 ---------+---------+---------+---------+---------+---------+ 240
    TCGCTTCCAGAGCGGTTCGAGCTACTTTAGCTCCTCCGTTTCGCGTTCGACGTTCCGTTC

 61 S  E  G  L  A  K  L  D  E  I  E  E  A  K  R  K  L  Q  G  K  80

                                                  sense primer
                                                  CTCTAGAGAAG

    GTGCAGGAACTAACCGACGCCAACAAAATGGCGTTCGCCAAGATTGGATCTCTAGAGAAG
241 ---------+---------+---------+---------+---------+---------+ 300
    CACGTCCTTGATTGGCTGCGGTTGTTTTACCGCAAGCGGTTCTAACCTAGAGATCTCTTC

 81 V  Q  E  L  T  D  A  N  K  M  A  F  A  K  I  G  S  L  E  K  100

       G6172
    ACTCGCCACAAGC

    ACTCGCCACAAGCTTATGCAAGATCTCGATGACGCTCAGGTCGACGTTGAGCGCGCGTGT
301 ---------+---------+---------+---------+---------+---------+ 360
    TGAGCGGTGTTCGAATACGTTCTAGAGCTACTGCGAGTCCAGCTGCAACTCGCGCGCACA

101 T  R  H  K  L  M  Q  D  L  D  D  A  Q  V  D  V  E  R  A  C  120

    TATGTGTGGCTTGAGAAAAAAGCAGAAAGGCTTCGCGACAAGATTATCGATGAATGGAGA
361 ---------+---------+---------+---------+---------+---------+ 420
    ATACACACCGAACTCTTTTTTCGTCTTTCCGAAGCGCTGTTCTAATAGCTACTTACCTCT

121 Y  V  W  L  E  K  K  A  E  R  L  R  D  K  I  I  D  E  W  R  140
```

```
    AAGAAGCACGATGACCTTGCTGCTGAACTCGATGCTGCTCAACGGGACAACCGCAACCTT
421 ---------+---------+---------+---------+---------+---------+ 480
    TTCTTCGTGCTACTGGAACGACGACTTGAGCTACGACGAGTTGCCCTGTTGGCGTTGGAA

141 K  K  H  D  D  L  A  A  E  L  D  A  A  Q  R  D  N  R  N  L  160

    TCCACTGATCTCTTCCGAGCCAAGACCGCCCAGGACGAGCTTGCCGAGCACCTTGAGAGC
481 ---------+---------+---------+---------+---------+---------+ 540
    AGGTGACTAGAGAAGGCTCGGTTCTGGCGGGTCCTGCTCGAACGGCTCGTGGAACTCTCG

161 S  T  D  L  F  R  A  K  T  A  Q  D  E  L  A  E  H  L  E  S  180

    GTACGACGTGAGAACAAGCAGCTTGCTCAAGAGGTGAAAGACCTTGCTGACCAACTCGGC
541 ---------+---------+---------+---------+---------+---------+ 600
    CATGCTGCACTCTTGTTCGTCGAACGAGTTCTCCACTTTCTGGAACGACTGGTTGAGCCG

181 V  R  R  E  N  K  Q  L  A  Q  E  V  K  D  L  A  D  Q  L  G  200

    GAAGGACGTTCCGCTCATGAACTCCAGAAAGGATGGCACGCCGTTTTGGGAAGTCGAAAA
601 ---------+---------+---------+---------+---------+---------+ 660
    CTTCCTGCAAGGCGAGTACTTGAGGTCTTTCCTACCGTGCGGCAAAACCCTTCAGCTTTT

201 E  G  R  S  A  H  E  L  Q  K  G  W  H  A  V  L  G  S  R  K  220

    GGGAAGAATCACAGCAAGGC
661 ---------+---------+ 680
    CCCTTCTTAGTGTCGTTCCG

221 G  K  N  H  S  K  A  227
```

(b) The nucleotide and derived amino acid sequences for the 5' end of KM35 (shown in the 5' to 3' orientation).

```
    GCGCTCAAGGGAGCCCAAAAAATCATTGNCCAACTCGAACAACGAATTCGTANCCTCGAA
  1 ---------+---------+---------+---------+---------+---------+ 60
    CGCGAGTTCCCTCGGGTTTTTTAGTAACNGGTTGAGCTTGTTGCTTAAGCATNGGAGCTT

  1 A  L  K  G  A  Q  K  I  I  X  Q  L  E  Q  R  I  R  X  L  E  20

    CAAGAACTTGATGGAGAACAGAGGCGTCACCAGGACACCGACAAGAACTGGCGCAAGTCC
 61 ---------+---------+---------+---------+---------+---------+ 120
    GTTCTTGAACTACCTCTTGTCTCCGCAGTGGTCCTGTGGCTGTTCTTGACCGCGTTCAGG

 21 Q  E  L  D  G  E  Q  R  R  H  Q  D  T  D  K  N  W  R  K  S  40

    GAGCGCCGTGTCAAGGAGGTCGAATTCCAACTGGAGGAAGACAAGAAGAATCAGGAAAGA
121 ---------+---------+---------+---------+---------+---------+ 180
    CTCGCGGCACAGTTCCTCCAGCTTAAGGTTGACCTCCTTCTGTTCTTCTTAGTCCTTTCT

 41 E  R  R  V  K  E  V  E  F  Q  L  E  E  D  K  K  N  Q  E  R  60

    CTCACCGAACTCATCGACAAACTCCAAGCCAAGCTCAAGGTGTTCAAGCGACAGGTTGAA
181 ---------+---------+---------+---------+---------+---------+ 240
    GAGTGGCTTGAGTAGCTGTTTGAGGTTCGGTTCGAGTTCCACAAGTTCGCTGTCCAACTT

 61 L  T  E  L  I  D  K  L  Q  A  K  L  K  V  F  K  R  Q  V  E  80

    GAGGCGGAAGAAGTTTCCGCTACCAACCTTGGCAAGTACCGTCAGCTTCAGGCTCAGCTC
241 ---------+---------+---------+---------+---------+---------+ 300
    CTCCGCCTTCTTCAAAGGCGATGGTTGGAACCGTTCATGGCAGTCGAAGTCCGAGTCGAG

100 E  A  E  E  V  S  A  T  N  L  G  K  Y  R  Q  L  Q  A  Q  L  100

    GATGATGCTGAGGAACGTGCCGATATTGCTGAAAACGCTCTGAGCAAAATGCGCAACAAG
301 ---------+---------+---------+---------+---------+---------+ 360
    CTACTACGACTCCTTGCACGGCTATAACGACTTTTGCGAGACTCGTTTTACGCGTTGTTC

101 D  D  A  E  E  R  A  D  I  A  E  N  A  L  S  K  M  R  N  K  120

    ATCCGTGCATCGGCNTCTGTTGGACCCAGGCCAGGGAGGACTCATGCAATCGGNCAGNTC
361 ---------+---------+---------+---------+---------+---------+ 420
    TAGGCACGTAGCCGNAGACAACCTGGGTCCGGTCCCTCCTGAGTACGTTAGCCNGTCNAG

                    CAACCTGGGTCCGGTCCCTCCTGAG
                      antisense primer G6171

121 I  R  A  S  A  S  V  G  P  R  P  G  R  T  H  A  I  G  Q  X  140
```

```
    GTCTGTNATACGAAACACCTCANTCGCCAGAGGAGGAGATTTCTAAGTNNAGNATCTGCT
421 ---------+---------+---------+---------+---------+---------+ 480
    CAGACANTATGCTTTGTGGAGTNAGCGGTCTCCTCCTCTAAAGATTCANNTCNTAGACGA

141 V  C  X  T  K  H  L  X  R  Q  R  R  R  F  L  S  X  X  S  A   160

    ATAATAACAGCCGTTTTGCTTGGCTATAACGTCTTCGATATTTCGCTCTTTGCGGTCACT
481 ---------+---------+---------+---------+---------+---------+ 540
    TATTATTGTCGGCAAAACGAACCGATATTGCAGAAGCTATAAAGCGAGAAACGCCAGTGA

161 I  I  T  A  V  L  L  G  Y  N  V  F  D  I  S  L  F  A  V  T   180

    TTANTCTTCAAGTCGTGGATAGCCGACACCTCTNCTGCTCTGTTCANTTGTTTGTCTCCA
541 ---------+---------+---------+---------+---------+---------+ 600
    AATNAGAAGTTCAGCACCTATCGGCTGTGGAGANGACGAGACAAGTNAACAAACAGAGGT

181 L  X  F  K  S  W  I  A  D  T  S  X  A  L  F  X  C  L  S  P   200

    NCNAATTCCAACTTCCC
601 ---------+------- 617
    NGNTTAAGGTTGAAGGG

201 X  N  S  N  F  G  206
```

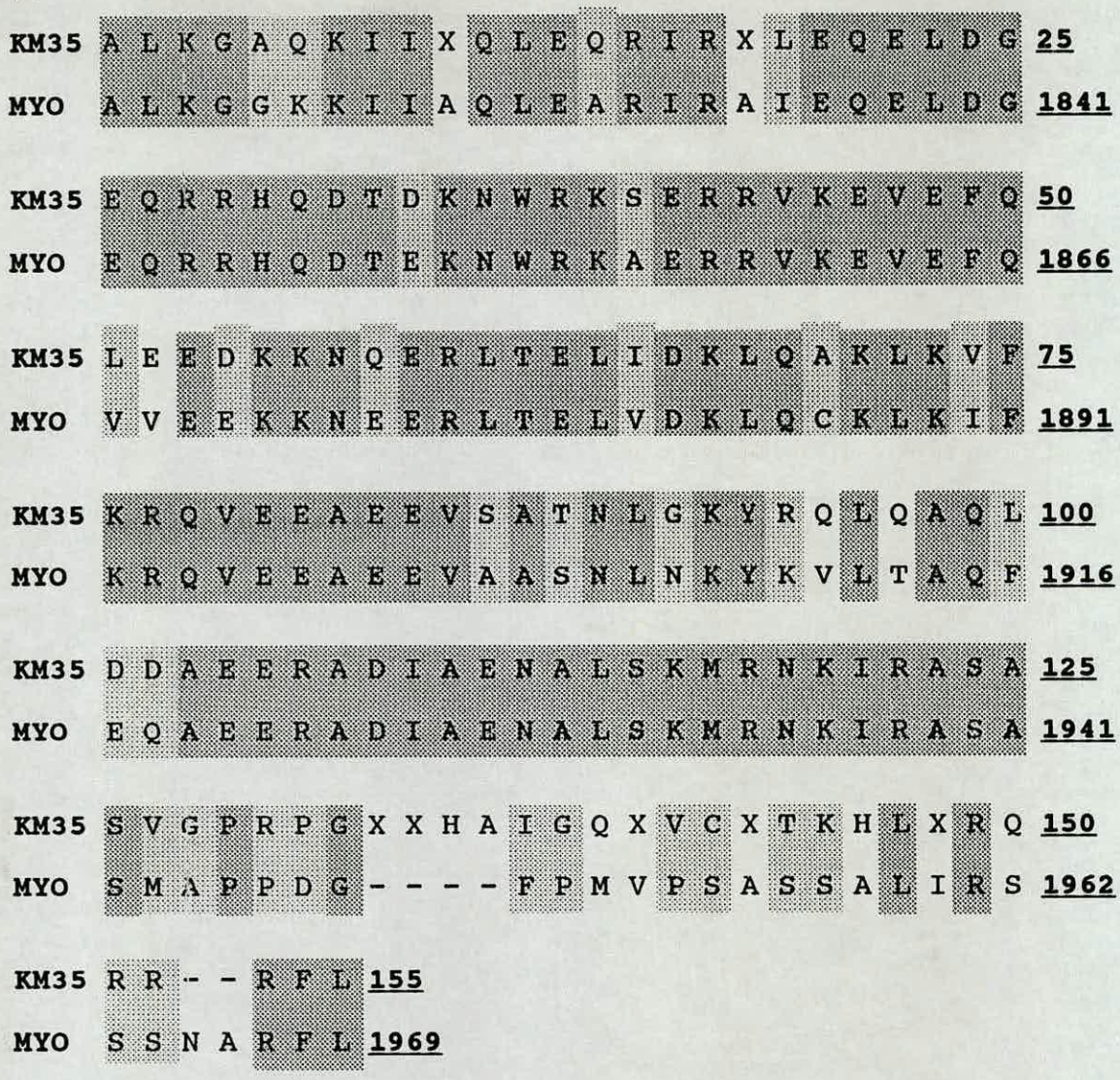
Figure 5.13

Alignment of the deduced amino acid sequence for the (a) 5' and (b) 3' ends of the KM35 insert with homologous segments from *C. elegans* myosin heavy chain A.

(a)

```
KM35 X N Y D E E A R E R Q A L A A Q V I T F E H E N D 25
MYO  R N Y D E E S R E R Q A L A A T A K N L E H E N T 1354

KM35 S L R D Q L A Q E S E A K A E L L R Q I R K Q N A 50
MYO  I L R E H L D E E A E S K A D L T R Q I S K L N A 1379

KM35 E I Q Q W K A R F E S E G L A K L D E I E E A K R 75
MYO  E I Q Q W K A R F D S E G L N K L E E I E A A K K 1404

KM35 K L Q G K V Q E L T D A N K M A F A K I G S L E K 100
MYO  A L Q L K V Q E L T D T N E G L F A K I A S Q E K 1429

KM35 T R H K L M Q D L D D A Q V D V E R A C Y - V W L 124
MYO  V R F K L M Q D L D D A Q S D V E K A A A Q V A F 1454

KM35 E K K A E R L R D K I I D E W R K K H D D L A A E 149
MYO  Y E K H R R Q F E S I I A E W K K K T D D L S S E 1479

KM35 L D A A Q R D N R N L S T D L F R A K T A Q D E L 173
MYO  L D A A Q R D N R Q L S T D L F K A K T A N D E L 1504

KM35 A E H L E S V R R E N K Q L A Q E V K D L A D Q L 298
MYO  A E Y L D S T R R E N K S L A Q E V K D L T D Q L 1529

KM35 G E - G R S A H E L Q K G W H A V L G S R K G K N 223
MYO  G E G G R S V A E L Q K I V R K L E V E K E E L Q 1554

KM35 H S 225
MYO  K A 1556
```

(b)



The deduced amino acid sequences of the 5' and 3' ends of the KM35 insert were aligned to similar regions of *C. elegans* myosin heavy chain A (MYO; Dibb *et al.*, 1988). The 5' end showed 65.6% identity (▩) and 74.1% conservation (▩) over 226 amino acids and the 3' end expressed 72.1% identity (▩) and 82.8% conservation (▩) over 157 amino acids. Dashes (-) indicates a gap in the amino acid sequence. The numbers correspond to the position of the adjacent amino acids in the respective peptide sequences.

Figure 5.14

Alignment of the derived nucleotide sequence for the 3' end of KM35 with homologous regions from *C. elegans* myosin heavy chain A (MYO) and *T. colubriformis* tropomyosin (TROP).

```
MYO    GCATTGAAGGGAGGAAAGAAGATCATTGCTCAATTGGAAGCCAGAATTCGTGCAATTGAA 9829
       ::   : :::::::      ::  ::::::: ::: : :::   ::::::::  :::
       GCGCTCAAGGGAGCCCAAAAAATCATTGNCCAACTCGAACAACGAATTCGTANCCTCGAA
KM35   ---------+---------+---------+---------+---------+---------+ 60
       CGCGAGTTCCCTCGGGTTTTTTAGTAACNGGTTGAGCTTGTTGCTTAAGCATNGGAGCTT


MYO    CAAGAATTGGATGGAGAACAGAGACGTCATCAAGACACCGAGAAGAACTGGAGAAAGGCT 9889
       :::::: : :::::::::::::: ::::: :: :::::::: ::::::::: : ::: :
       CAAGAACTTGATGGAGAACAGAGGCGTCACCAGGACACCGACAAGAACTGGCGCAAGTCC
KM35   ---------+---------+---------+---------+---------+---------+ 120
       GTTCTTGAACTACCTCTTGTCTCCGCAGTGGTCCTGTGGCTGTTCTTGACCGCGTTCAGG


MYO    GAAAGACGTGTCAAGGAGGTTGAATTCCAAGTTGTCGAGGAGAAGAAGAATGAAGAAAGA
       ::  : :::::::::::::: ::::::::: : :  :: :: ::::::::: : ::::::
       GAGCGCCGTGTCAAGGAGGTCGAATTCCAACTGGAGGAAGACAAGAAGAATCAGGAAAGA
KM35   ---------+---------+---------+---------+---------+---------+ 180
       CTCGCGGCACAGTTCCTCCAGCTTAAGGTTGACCTCCTTCTGTTCTTCTTAGTCCTTTCT


MYO    CTGACGGAATTGGTTGACAAGCTTCAATGCAAACTTAAGATCTTCAAGAGACAGGTTGAG 10009
       :: :: ::: :  : ::::: :: :::  ::: :: ::: : :::::: ::::::::::
       CTCACCGAACTCATCGACAAACTCCAAGCCAAGCTCAAGGTGTTCAAGCGACAGGTTGAA
KM35   ---------+---------+---------+---------+---------+---------+ 240
       GAGTGGCTTGAGTAGCTGTTTGAGGTTCGGTTCGAGTTCCACAAGTTCGCTGTCCAACTT


MYO    GAGGC 10014
       :::::
       GAGGC.....................................................
KM35   ----- 245
       CTCCG.....................................................


MYO          GGAGGAAGTTGCCGCCAGCAACTTGAACAAGTACAAGGTTCTCACTGCTCAATTT 10145
             ::: :::::: :::: : :::: :   :::::::      ::    :::::  :
       .....GGAAGAAGTTTCCGCTACCAACCTTGGCAAGTACCGTCAGCTTCAGGCTCAGCTC
KM35         ---+---------+---------+---------+---------+---------+ 300
       .....CCTTCTTCAAAGGCGATGGTTGGAACCGTTCATGGCAGTCGAAGTCCGACTCGAG


MYO    GAACAAGCTGAAGAGAGAGCCGATATTGCTGAAAATGCCCTG-TCAAAGATGCGTAACAAG 10205
       ::  : ::::: ::  : ::::::::::::::::: :: :::  :::: ::::: ::::::
       GATGATGCTGAGGAACGTGCCGATATTGCTGAAAACGCTCTGAGCAAA-ATGCGCAACAAG
KM35   ---------+---------+---------+---------+-------- -+---------+ 360
       CTACTACGACTCCTTGCACGGCTATAACGACTTTTGCGAGACTCGTTT-TACGCGTTGTTC
                    ::::::::: :::::::::::: ::::::::: ::::::::::::
TROP                TTGCACGGCTGTAACGACTTTTGCGGGACTCGTTT-TACGCGTTGTTC 219
```

```
MYO   ATTCGTGCATCAGCTTC                                              10222
      :: :::::::: :: ::
      ATCCGTGCATCGGCNTCTGTTGGACCCAGGCCAGGGNGGANTCATGCAATCGGNCAGNTC
KM35  ---------+---------+---------+---------+---------+---------+ 420
      TAGGCACGTAGCCGNAGACAACCTGGGTCCGGTCCCNCCTNAGTACGTTAGCCNGTCNAG
      :::::::::::::: :::::::::::::: :::::: ::: : :::::::::: ::: ::
TROP  TAGGCACGTAGCCGAAGACAACCTGGGTC-GGTCCCTCCTGATTACGTTAGCCGGTCGAG 160

      GTCTGTNATACGAAACACCTCANTCGCCAGAGGAGGAGATTTCTAAGTNNAGNATCTGCT
KM35  ---------+---------+---------+---------+---------+---------+ 480
      CAGACANTATGCTTTGTGGAGTNAGCGGTCTCCTCCTCTAAAGATTCANNTCNTAGACGA
        :::: :: :::: ::::::: :::::::::::::::::::: ::::   ::  ::: ::
TROP  TCGACAGTACGCTTCGTGGAGTAAGCGGTCTCCTCCTCTAAAAATTC-GGTCGAAGATGA 101

      ATAATAACAGCCGTTTTGCTT--GGCTATAACGTCTTCGATATTTCGCTCTTTGCGGTCACT
KM35  ---------+---------+-  --------+---------+---------+---------+ 540
      TATTATTGTCGGCAAAACGAA--CCGATATTGCAGAAGCTATAAAGCGAGAAACGCCAGTGA
      ::: : ::::::::::::::  :::::: :::::::::: ::::::: ::::::::::::
TROP  TATGACTGTCGGCAAAACGAACCCCGATACTGCAGAAGCTA-AAAGCGACAAACGCCAGTGA 40

      TTANTCTTCAAGTCGTGGATAGCCGACACCTCTNCTGCTCTGTTCANTTGTTTGTCTCCA
KM35  ---------+---------+---------+---------+---------+---------+ 600
      AATNAGAAGTTCAGCACCTATCGGCTGTGGAGANGACGAGACAAGTNAACAAACAGAGGT
      ::: :  ::::::::::  : ::: ::    ::  ::
MYO   AATCACGAGTTCAGCATATGTCGTCTCCACAGCCGATAC                        1

      NCNAATTCCAACTTCCC
KM35  ---------+------- 617
      NGNTTAAGGTTGAAGGG
```

The nucleotide sequence for the 3' end of the KM35 insert was aligned to similar regions of *C. elegans* myosin heavy chain A (MYO) and *T. colubriformis* tropomyosin (TROP). The nucleotide sequence for KM35 3' end is 617 bp in length. For KM35, the upper nucleotide sequence corresponds to the sense strand and reads 5' to 3'. The lower KM35 sequence is the antisense strand and runs 3' to 5'. In the nucleotide sequence, 'N' denotes A, C, G or T. The KM35 sense strand (bases 1 to 453) showed 74.2% identity (:) with *C. elegans* myosin heavy chain A (Dibb *et al.*, 1989). Also, the antisense strand of KM35, reading from bases 576 to 314, showed 85.7% identity (:) to a similar region in *T. colubriformis* tropomyosin mRNA (Frenkle *et al.*, 1989). The symbol (-) indicates a gap in the nucleotide sequence. The numbers correspond to the position of the adjacent base in the respective nucleotide sequence.

5.3 DISCUSSION

Prior to this thesis, very little research had been carried out to define the nature of the proteins excreted and secreted by *T. vitrinus*. Selected biochemical enzyme analysis in chapter three revealed that adult *T. vitrinus* ES contained AChE and proteinases. The aim of the work described in this chapter was to isolate cDNA fragments encoding ES proteins of adult *T. vitrinus* and to attempt to define the proteins by sequence analysis.

Western blot analysis of ES proteins confirmed that the rabbit antibodies raised against adult *T. vitrinus* ES recognised the major ES proteins (figure 5.1). Although distinct banding was evident the profile was blurred which could indicate that the ES proteins were glycosylated and that antibody was directed to the carbohydrate epitopes. Foreign peptides expressed as β-galactosidase fusions in λgt11 are not glycosylated and, hence, an antiserum recognising a propensity of carbohydrate epitopes would have limited utility for library screening. However, the recognition profile was unaffected by periodate treatment of the blot strips prior to antibody probing (figure 5.1 (b), lanes 1 and 2). Periodate treatment blocks carbohydrate epitopes with the result that the antibody recognition profile is restricted to protein epitopes (Woodward, Young and Bloodgood, 1985).

Using this serum, a λgt11 cDNA expression library was screened and 14 immunopositive clones harbouring recombinant inserts were isolated. The foreign DNA inserts from 10 of these clones were subsequently sequenced.

Lysogen analysis of the fusion proteins expressed by the recombinants met with limited success. IPTG induction did indeed result in the expression of a 116 kDa protein in native λgt11, the expected size for mature β-galactosidase, confirming the induction process was working (figure 5.9) and fusion proteins of approximately the anticipated size were faintly evident following SDS-PAGE analysis upon induction of KM1 and KM26. However, proteins of the appropriate size were not clearly demonstrated in the remaining recombinants, possibly indicating that the fusion peptide was unstable and degraded. Degradation was indicated by Western blot analysis of the fusion expressed by KM1 where clear immunoreactive peptides were evident as a ladder below the expected size of 180 kDa (figure 5.10). However, by

this method analysis did indicate that a KM19 fusion protein was expressed by the corresponding lysogen even though it could not be seen by SDS-PAGE and Coomassie staining. Prior to use, anti-*E. coli* antibodies were extensively absorbed from the antiserum by absorption with *E. coli* lysate proteins and whole cells, and this process may have non-specifically removed antibody with specificity for *T. vitrinus* ES proteins. This, combined with the poor level of fusion protein induction observed, may have contributed to the apparent non-recognition of the recombinants when analysed by the rabbit anti-*T. vitrinus* ES serum.

Seven of the clones contained DNA fragments that showed no significant homology at either their nucleotide or peptide level, with any of the recorded sequences currently in the computer sequence analysis databases. Nevertheless, the unknown proteins encoded by these DNA fragments may be important to the survival of the parasite *in vivo* and also may stimulate a protective response in the host. A coupled *in vitro* transcription/translation assay using bacterial extracts could be used to provide information on the anticipated size of the fusion protein encoded and full-length cDNAs could be generated by RACE-PCR.

The largest insert was present in clone KM1 (1.33 kb) and the peptide encoded by this DNA fragment was found to be very similar to the C-terminal region of heavy chain myosin, a protein normally found in muscle (figure 5.6). However, some controversy as to whether myosin is a true ES component seems to reign from when λgt11 libraries have been immunoscreened using sera specific for other proteins, by workers, here, at the Moredun Research Institute, myosin has been isolated. Western blot analysis of the lysogen prepared from KM1 suggests that the expressed recombinant protein from this clone was, in fact, not recognised exclusively by the anti-ES serum, as the protein also was recognised by normal rabbit serum (figure 5.10). However, it is interesting that parasite myosin has been selected as a major antigenic protein which is recognised by sera from human hosts infected by *B. malayi* (Werner *et al.*, 1989), *Wuchereria bancrofti* (Dissanayake, Xu and Piessens, 1992) and *S. mansoni* (Newport *et al.*, 1987). Since myosin is usually associated with muscle protein, it is not known how myosin might become exposed to the host immune system. Werner *et al.* (1989) suggest that it may reflect on degradation of

the worm on death (population turnover) or, alternatively, the protein itself may be broken down and excreted during the normal functioning of the microfilaria (physiological turnover). The tail of myosin forms an α-helix which interacts with another myosin tail in a helical arrangement, forming a structure known as a coiled coil. Werner *et al.* (1989) note that this motif is shared by several proteins, including the M protein of the bacterium, *Streptococcus pyogenes* (Hollingshead, Fischetti and Scott, 1986). This coiled coil of the M protein binds to serum factor H inhibiting the alternative pathway of complement activation and so, the motif acts as a means of evading immune recognition (Horstmann *et al.*, 1988; cited in Werner *et al.*, 1989). Based on this knowledge, Werner *et al.* (1989) also proposed that the parasite myosin may be an important protein in the course of a parasitic infection. Another muscle protein, paramyosin, has also been identified as an immunodominant protein and is recognised by sera from hosts infected with *O. volvulus* (Donelson *et al.*, 1988) and *S. mansoni* (Lanar *et al.*, 1986; Pearce *et al.*, 1988). Furthermore, in the latter case, paramyosin has been shown to induce protective immunity against the parasite (Pearce *et al.*, 1988).

KM33 harboured an insert that encoded a very similar peptide to the yolk proteins, vitellogenins, of *C. elegans* (figure 5.8). *C. elegans* possesses six genes that encode vitellogenins (*vit* genes), the coding regions of which are highly conserved (Kimble and Ward, 1988). These proteins are only expressed abundantly in the intestinal cells of the adult hermaphrodite stage of *C. elegans* and not in the larval stages or in the male worm (Kimble and Ward, 1988; Hogdkin, 1988). Once, synthesised, the yolk proteins are secreted into the pseudocoelum of the worm and are taken up by the gonad to be incorporated into the developing oocytes (Hogdkin, 1988). The vitellogenin-like protein isolated here, was recognised by antiserum raised to the ES of adult *T. vitrinus* proteins. It is not known why vitellogenin may be present in the ES of adult *T. vitrinus*. Recently however, Willadsen *et al.* (1994) isolated an 86 kDa protein by fractionation from the cattle tick, *B. microplus*, that showed high homology to vitellogenins from other species. Furthermore, when the purified 86 kDa protein was used in vaccine trials, with sheep as a model tick host, the

numbers of ticks engorging and the weight of the ticks were found to be reduced (Willadsen *et al.*, 1994).

Immunopositive KM19 possessed a recombinant insert of 398 bp. On analysis of its respective sequence it was found that a peptide, encoded in the opposite orientation to β-galactosidase showed significant homology to several types of serpins. The bacteriophage, λgt11, is designed so as to express foreign inserted DNA as a β-galactosidase fusion protein (Young and Davis, 1983). However, it has been previously recorded that antigenic proteins may sometimes be expressed by λgt11 even when the inserts were in the opposite orientation and not fused to β-galactosidase. For example, in a screening of a yeast genomic library in λgt11 for *TOP*2 gene, five positives were selected, three of which contained inserts orientated so that the direction of transcription was opposite to that of β-galactosidase (Goto and Wand, 1984). The inserts were unlikely to possess sequences that could be used as promoters by *E. coli* RNA polymerase, therefore, the authors (Goto and Wand, 1984) proposed that the expression of these fragments was from a late phage λ promoter in the vector. Similarly, immunopositive clones selected from a *Plasmodium falciparum* genomic DNA λgt11 library resulted in the isolation of phage harbouring recombinant proteins that were expressed in the opposite direction to β-galactosidase (Dame *et al.*, 1984). The same phenomenon was also demonstrated in clones selected from a *Dirofilaria immitis* λgt11 library (Grandea III *et al.*, 1989).

Chirala (1986) sequenced the lac operon and phage junction in λgt11. The *lom* region of the lambda and lac operon are transcribed in opposite directions in λgt11 (Daniels *et al.*, 1983; Chirala, 1986). In the direction opposite to that of *lac* translation, the sequence had one translational open reading frame which continued past the *Eco*R1 site into the insert: the other reading frames, including that of the Lom protein, ended prior to this point (Daniels *et al.*, 1983; Chirala, 1986). Therefore, this or some other upstream promoter on the opposite strand to β-galactosidase may be responsible for transcription (Daniels *et al.*, 1983; Chirala, 1986). Dame *et al.* (1984) and Grandea III *et al.* (1989) also isolated antigenic λgt11 clones where the inserts were in the same orientation as β-galactosidase but were one base out of frame to be

expressed as part of the fusion protein. However, such a situation was not observed in the present study. The analysis of KM19 is described in detail in chapter six

Western blot analysis of the recombinant lysogen from KM19 revealed the presence of a 110 kDa protein which appeared only on induction of the host cells (figure 5.10). If the recombinant insert in KM19 was expressed as a fusion protein of β-galactosidase, a protein of 135 kDa would be expected to be synthesised on induction. Sequence analysis of KM19 suggests that the recombinant protein is expressed in the opposite direction to β-galactosidase. Therefore, this 110 kDa may be a foreign protein encoded for by the KM19 DNA insert, in fusion with another λgt11 protein encoded downstream of *lacZ* on the opposite strand.

The adult *T. vitrinus* λgt11 cDNA library was also screened with rabbit antiserum that was raised against purified sAChE from *T. colubriformis*, a nematode closely related to *T. vitrinus*. Western blot analysis of adult *T. colubriformis* homogenate and ES proteins, separated by SDS-PAGE under reducing conditions, demonstrated that the anti-adult *T. colubriformis* sAChE serum recognises protein bands of 110, 90 and 66 kDa in the homogenate and a band of 66 kDa in the ES (G. Griffiths, University of Nottingham, UK, personal communication). Analysis *of T. vitrinus* homogenate and ES, using the same conditions, showed that in both cases the anti-*T. colubriformis* sAChE serum recognised proteins that did not migrate very far into the gel (figure 5.1 (b), lanes 4 and 5). In the presence of SDS and β-mercaptoethanol, non-covalent interactions in native proteins are disrupted and disulphide bonds within the proteins are reduced. If the protein which is recognised strongly by the anti-*T. colubriformis* sAChE serum is AChE, then the results suggests that *T. vitrinus* AChE is either made up of subunits, or a single molecule of very high molecular weight. Most parasites, including *T. colubriformis*, possess AChEs made of catalytic subunits in the region of 60-100 kDa (Rhoads, 1984; Griffiths and Pritchard, 1994 a).

On immunoscreening of the adult *T. vitrinus* cDNA in λgt11 with the anti-sAChE serum, a single positive clone (KM35), containing an insert of 2 kb in size, was selected. Sequence analysis of KM35 concluded that the insert encoded a myosin that had high identity to *C. elegans* myosin heavy chain A (figure 5.13). Given that

the antiserum used for immunoscreening was specifically raised against purified *T. colubriformis* sAChE, it was expected that the KM35 recombinant DNA fragment would encode an AChE. Isolation of a myosin clone may be due to non-specific recognition, as found in the previous immunoscreen with the anti-ES serum, though the reactivity of the KM35 recombinant lysogen with normal rabbit serum was not assessed. Alternatively, these two proteins may share immunologically related domains which is not apparent from the primary sequence.

Nucleotide sequence analysis of the 3' region of the KM35 insert, which was 617 bp in length, revealed that from nucleotide 1 - 377 the sequence was 75% identical to the corresponding region of the *C. elegans myo*-3 gene. Also, reading along the antisense strand from nucleotide 617 - 314, the sequence was 86% identical, over 266 bases, with a 5' untranslated sequence from *T. colubriformis* tropomyosin mRNA. Initially, it was assumed that the KM35 insert comprised of two different fragments of DNA, that had ligated together during the cloning procedure. However, there is a section of the insert, 63 bp in length (bp 314 - 377), where the sense strand matches the *C. elegans* myosin gene and the antisense sequence corresponds to *the T. colubriformis* tropomyosin mRNA. This suggests that the two sequences may share a common overlapping gene locus. Northern blot analysis, using the individual myosin and tropomyosin portions of the KM19 DNA fragment as probes, might help to confirm this.

Although several ES proteins were recognised by serum from a lamb which had been immunised against *T. vitrinus* infection by repeated infection with the parasite, none of the recombinants isolated here using the rabbit anti-ES serum were unequivocally recognised by the hyper immune lamb serum (not shown). Immunity to *T. vitrinus* is expressed in the intestinal mucosa and infection stimulates local mucosal antibody responses (see chapter one). In the lamb, circulating antibody titres to ES proteins may be too low, or non-existent, to enable detection of these recombinants. Efferent lymph sampled from GI tract lymphatics may provide a more appropriate probe for isolating recombinants encoding ES proteins with relevance to the stimulation of mucosal immunity.

In summary, immunoscreening of an adult *T. vitrinus* cDNA λgt11 expression library, with antiserum raised against the parasite ES, resulted in the isolation of 10 immunopositive clones which were subsequently sequenced. Seven of the clones contained inserts that did not match any of the sequences currently catalogued in the computer databases. The three other clones contained inserts that encoded myosin, vitellogenin and a serpin like peptide. Immunoscreeing of the expression library with anti-*T. colubriformis* sAChE serum selected a single clone, that contained a myosin encoding recombinant DNA fragment.

Of particular interest was clone KM19 which harboured a insert that encodes a serpin-like protein, a molecule not previously identified in the ES of parasitic nematodes. Serine proteinases are involved in several host immune effector mechanisms and inhibition of one or more of these enzymes may be one way that *T. vitrinus* worms can evade the hosts immune system. Given the potential importance of this molecule to *T. vitrinus*, KM19 was chosen for further characterisation, as will be described in chapter six.

Chapter six

Characterisation of the cDNA from the immunopositive clone (KM19) expressing a serpin-like peptide

6.1 INTRODUCTION

Immunoscreening of an adult *T. vitrinus* cDNA λgt11 library with anti-adult *T. vitrinus* ES serum resulted in the isolation of several immunopositive clones, including a clone (KM19) that harboured a cDNA fragment encoding a serpin-like protein (chapter five). Given the potential importance of a putative secretory serpin to the survival of *T. vitrinus* in the host (see discussion), it was decided to concentrate the final part of the research for this thesis on characterisation of the KM19 cDNA insert.

6.1.1 Serpins

The serpin superfamily consists of over 40 serpins, widely distributed throughout species (reviewed by Travis *et al.*, 1990, Gettins *et al.*, 1992 and Marshall, 1993). The majority of serpins are involved in the regulation of proteolytic processes which are mediated by serine proteases, such as blood coagulation, the complement cascade, fibrinolysis and phagocytosis, though some serpins (for example, ovalbumin) lack inhibitory ability (reviewed by Potempa, Korzus and Travis, 1994). Despite the great evolutionary divergence in the primary structure and functions of serpins, the overall secondary and tertiary structures appear to be highly conserved (Marshall, 1993; Potempa, Korzus and Travis, 1994). Serpins are composed of a single chain of >350 amino acid residues and contain varied amounts of glycosylation and disulphide bonding. The tertiary structure is a highly ordered globular arrangement with nine α-helical regions and three β-sheets folded in a 'stressed' position. As an inhibitor, a serpin interacts with the target proteinase in a 1:1 molar ratio. The active centre is located within a loop structure, 30-40 amino acids upstream from the protein's carboxyl-terminus and is exposed to the surface: the reactive site amino acid is termed P_1 (Gettins *et al.*, 1992). Carbohydrate side chains are unimportant for the inhibition process (Travis *et al.*, 1990). The amino acids present in the reactive loop vary amongst serpins and minor changes can result in a substantial effect on the specificity of the inhibitor (Gettins *et al.*, 1992). For example, the naturally occurring mutation of Met to Arg at the reactive site P_1 amino

acid in α_1-antitrypsin (α_1-AT) changes its specificity from elastase to thrombin (known as Pittsburgh α_1-AT; Gettins *et al.*, 1992).

6.1.2 Technique for isolation of the 5' and 3' end cDNA sequences for KM19

In order to isolate the full length cDNA that encodes the KM19 cDNA fragment, PCR amplification of polyadenylated cDNA was applied, using primers specifically directed to the KM19 insert. This technique has successfully been utilised by colleagues at the Moredun Research Institute, Edinburgh, to isolate full length cDNAs encoding a variety of proteins from other ovine GI nematodes.

Initially, attempts were made to amplify only the 5' end of the cDNA by using the sense primer, SL1 (see section 4.1.3) and an antisense primer directed towards the KM19 insert. For amplification of the 3' end of the gene, primers directed towards the sense strand of KM19 and the antisense of the polyadenylated tail were employed. This technique was very similar to the procedure used in chapter four for amplifying adult *T. vitrinus* cDNA fragments encoding AChE. However, in this case, the KM19 primers were based on a known *T. vitrinus* cDNA sequence allowing the PCR amplifications to be carried out at high stringency.

6.2 RESULTS

6.2.1 Sequence analysis of the insert cDNA from immunopositive KM19

As detailed in section 5.2.6, translation of the KM19 nucleotide sequence in one of the three open reading frames in the antisense orientation to β-galactosidase, resulted in the identification of a peptide that showed significant homology to several types of serpins.

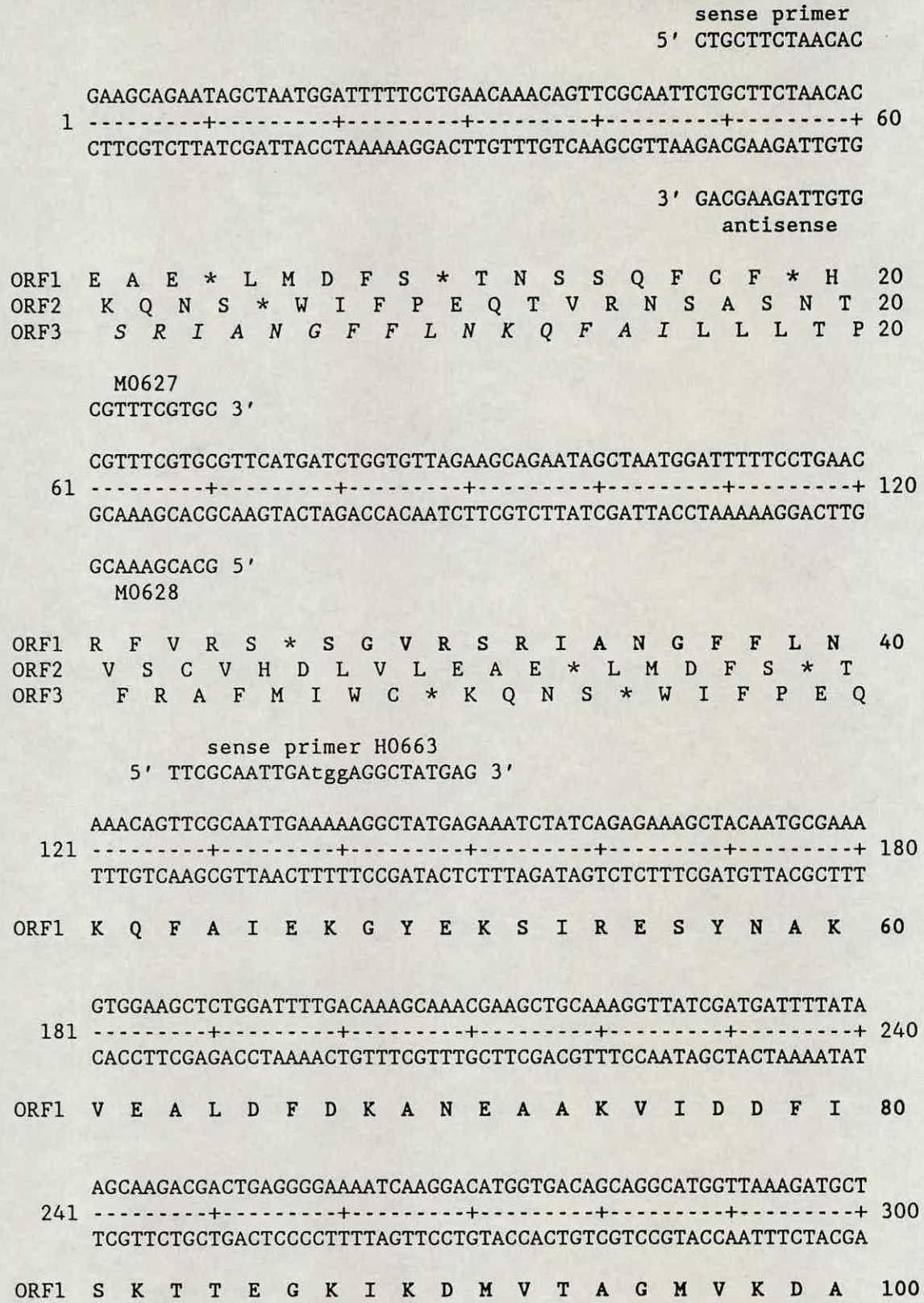
The complete nucleotide sequence as well as the derived amino acid sequences in all three open reading frames for the KM19 insert cDNA are shown in figure 6.1 (in the reverse complement of the determined sequence). Analysis revealed that the first 47 bp of the sequence were repeated at 89-135 bp and the sequence from 45-61 bp was the reverse complement of the 98-82 bp. The serpin-like peptide was 107 amino acids in length and read from nucleotide 79. Translation of the first 78 bp in the same open reading frame (ORF1) as the serpin-like peptide produced several stop codons. However, translation of the sequence, reading from the third nucleotide (ORF3), gave a straight open reading frame for 28 amino acids (86 bp). The first 15 amino acids of this peptide was actually repeated in ORF1 from amino acids 31 to 45.

The KM19 serpin-like peptide shared best homology with human leukocyte elastase inhibitor (LEI; Remold-O'Donnell, Chin and Alberts, 1992; figure 6.2) with 33.3% identity and 57.8% conservation over 104 amino acids. At least 20 other serpin molecules also showed 28 to 32% identity with KM19. Huber and Carell (1989) aligned 20 serpin sequences and identified 51 positions where there was amino acid conservation. In LEI, 49 of these 51 residues are conserved. The region of LEI which aligns to the KM19 serpin peptide contains 15 of these conserved amino acids (see figure 6.2). Ten of these amino acid residues are also conserved within the KM19 serpin (positions 57, 76, 84, 88, 106, 108, 110, 114, 118, 128 in the KM19 serpin). At position 49 in the KM19 serpin a Tyr is substituted for a Phe residue. This substitution is observed also in some serpins such as α_1-AT (Loebermann *et al.*, 1984) and plasminogen activator inhibitor 2 (PAI-2; Ye *et al.*, 1989). At positions 38 and 123 of the KM19 serpin a Phe is substituted for a Tyr and an Arg is substituted for a Thr respectively. The former deviation is found in several other serpins

including in α_1-AT (Loebermann *et al.*, 1984) and PAI-2 (Ye *et al.*, 1989) and the latter appears to occur solely in PAI-2 (Ye *et al.*, 1989). At position 77 of the KM19 serpin, an Asn was replaced with Asp and this change is also seen in angiotensinogen (Fukamizu *et al.*, 1990). The absence of a Leu between positions 104 and 105 of the KM19 serpin was not observed in any of the other serpin peptide sequences used for comparison.

Figure 6.1

Nucleotide sequence of the cDNA from immunopositive KM19.



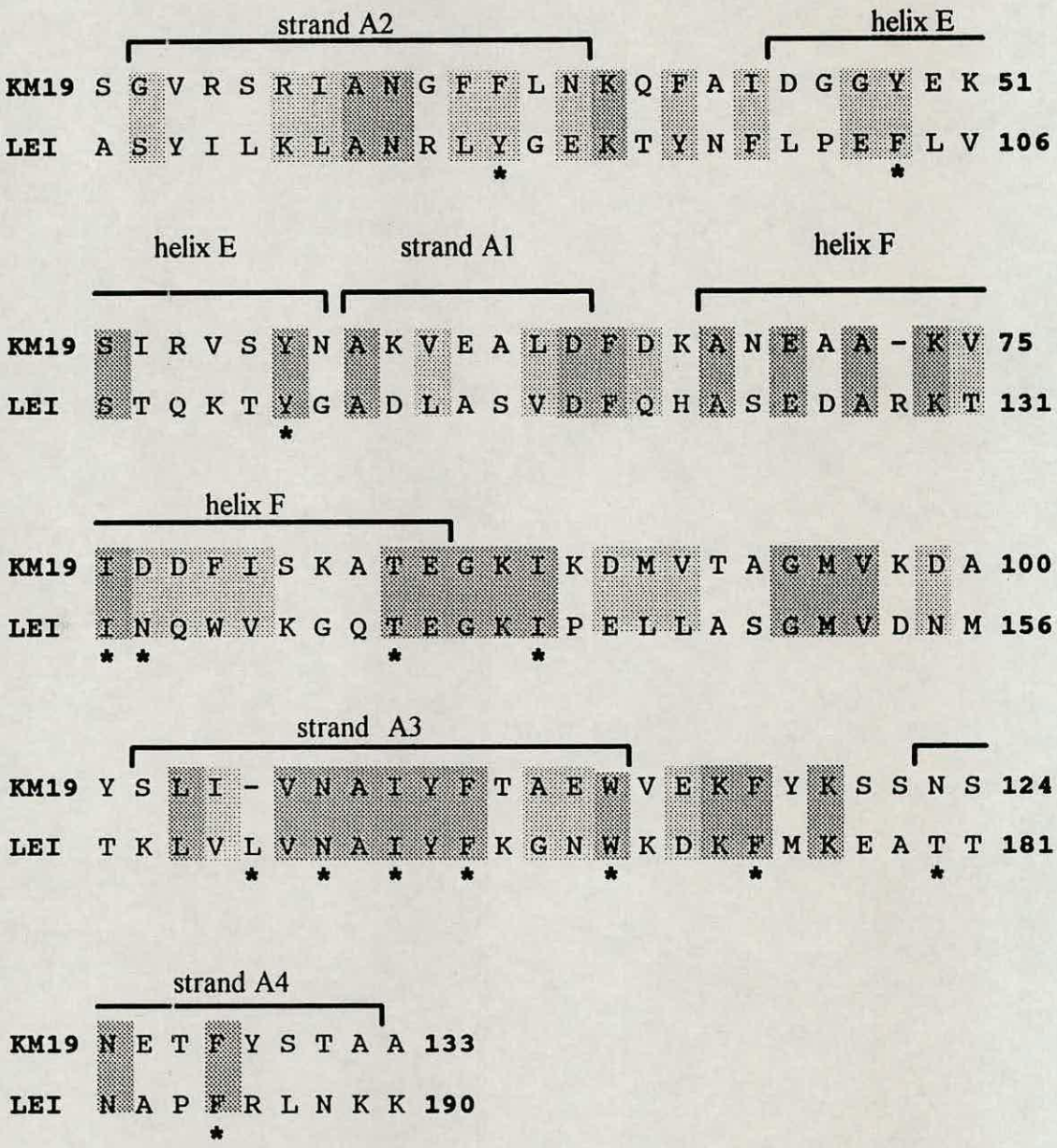
```
    TACTCCCTTATTGTCAATGCCATCTATTTCACTGCTGAATGGGTGGAAAAGTTCTACAAA
301 ---------+---------+---------+---------+---------+---------+ 360
    ATGAGGGAATAACAGTTACGGTAGATAAAGTGACGACTTACCCACCTTTTCAAGATGTTT

                                3' GTGACGACTTACCCACCTTTTCAA 5'
                                    antisense primer H0664

ORF1  Y  S  L  I  V  N  A  I  Y  F  T  A  E  W  V  E  K  F  Y  K  120


    AGTTCCAATTCAAACGAGACATTTTATAGCACAGCAGC
361 ---------+---------+---------+-------- 398
    TCAAGGTTAAGTTTGCTCTGTAAAATATCGTGTCGTCG

ORF1  S  S  N  S  N  E  T  F  Y  S  T  A  A  133
```

The cDNA insert in clone KM19 was 398 bp in length and gave an open reading frame (ORF1) encoding 107 amino acids (shown in bold), from nucleotide 79, when the nucleotide sequence was translated in the antisense orientation. The possibility of insert cDNA being expressed in this orientation as a non-β-galactosidase fusion was discussed in chapter five. The first 120 nucleotides were also translated in the two other possible open reading frames for this orientation, commencing from base 2 (ORF2) and base 3 (ORF3) respectively. Stop codons in the peptide sequences are indicated by an asterisk (*). The first and last nucleotide or amino acid in each line is numbered.

The oligonucleotide primers, two sense (M0627 and H0663) and two antisense (M0628 and H0664), which were designed to regions of the KM19 sequence are shown. Primer H0663 was synthesised after initial sequence analysis and bases 12-14 of this primer read TGG instead of AAA, as determined by the further sequencing. A PCR product generated by amplification of DNA extracted from *T .vitrimus* cDNA using primers M0627 and H0664 (section 6.2.7) had the identical nucleotide sequence to the sequence between these primers shown above.

Figure 6.2

Alignment of KM19 and human leucocyte elastase inhibitor (LEI) peptide sequences.



The KM19 serpin-like peptide was aligned with a similar section of human LEI (Remold-O'Donnell, Chin and Alberts, 1992). The numbers correspond to the position of the last amino acid in each line within the peptide sequence. The two peptides showed 33.3% identity (▒) and 57.8% conservation (▒). Dashes (-) represent gaps in the KM19 sequence. Asterisks (*) denote residues present in LEI that are categorised as conserved in the larger serpin superfamily. Structural regions, as based on α_1-antitrypsin (Huber and Carrell, 1989), are indicated.

6.2.2 Northern and genomic Southern blot analysis of KM19

Adult *T. vitrinus* polyadenylated RNA (mRNA) was isolated (section 2.11.5) and a Northern blot membrane was prepared as described in section 2.11.17. Isolated adult *T. vitrinus* genomic DNA (section 2.11.3) was restricted with either *Bam*HI, *Eco*RI or *Hind*III (section 2.11.13). Following restriction, an aliquot of each digest (1/20) was analysed by agarose (0.8%, w/v) gel electrophoresis (section 2.11.8) to ensure digestion was complete (figure 6.3). The remaining digested DNA was electrophoresed and Southern blotted onto nylon membrane, as detailed in section 2.11.16.

The insert cDNA from KM19 was PCR amplified (section 2.11.7) using the λ directed oligonucleotide primers, 514N and 515N (see table 2.2). The amplified product was electrophoresed through an agarose (0.8%, w/v) gel, after which, the DNA was excised and was purified by gencleaning (section 2.11.9). This was followed by a phenol:chloroform extraction (section 2.11.1) and ethanol precipitation (section 2.11.2). An aliquot of the purified KM19 fragment was electrophoresed and Southern blotted onto membrane (section 2.11.16) to act as a positive control.

The probe was prepared by PCR amplification of the purified KM19 insert, using KM19 primers H0663 and H0664 (figure 6.1), in which the majority of dCTP was in the form of [^{32}P]α-dCTP (section 2.11.19). The Northern and Southern blots were hybridised with the KM19 ^{32}P-labelled probe, following which, the blots were washed at 60°C in 0.1x (v/v) SSC, containing 1% (w/v) SDS (section 2.11.19).

The Northern blot showed that the KM19 insert hybridised to mRNA that was of approx. 1.2 kb in length (figure 6.4). In the Southern blot analysis, the probe hybridised strongly to a single band of approx. 800 bp in the *Eco*RI digested genomic DNA (figure 6.5, lane 2). In the *Hind*III DNA digested track, a faint band of approx. 4.2 kb was observed (figure 6.5, lane 1). No distinct banding was evident in the *Bam*HI digested track although a smear was evident at a high molecular weight size (figure 6.5, lane 3). The probe hybridised also to the purified KM19 fragment (figure 6.5, lane 4), as was expected.

Figure 6.3

Agarose gel electophoresis profile of enzyme restricted adult *T. vitrinus* genomic DNA.

Genomic DNA was extracted from adult *T. vitrinus* and aliquots (2-5 μg) were digested with either *Bam*HI, *Eco*RI or *Hind*III. Following digestion, 1/20 of the samples were analysed by agarose (0.8%, w/v) gel electrophoresis.

Lanes:

M - DNA markers

*Bam*HI, *Eco*RI and *Hind*III digested adult *T. vitrinus* genomic DNA are shown in Lanes 1, 2 and 3 respectively.

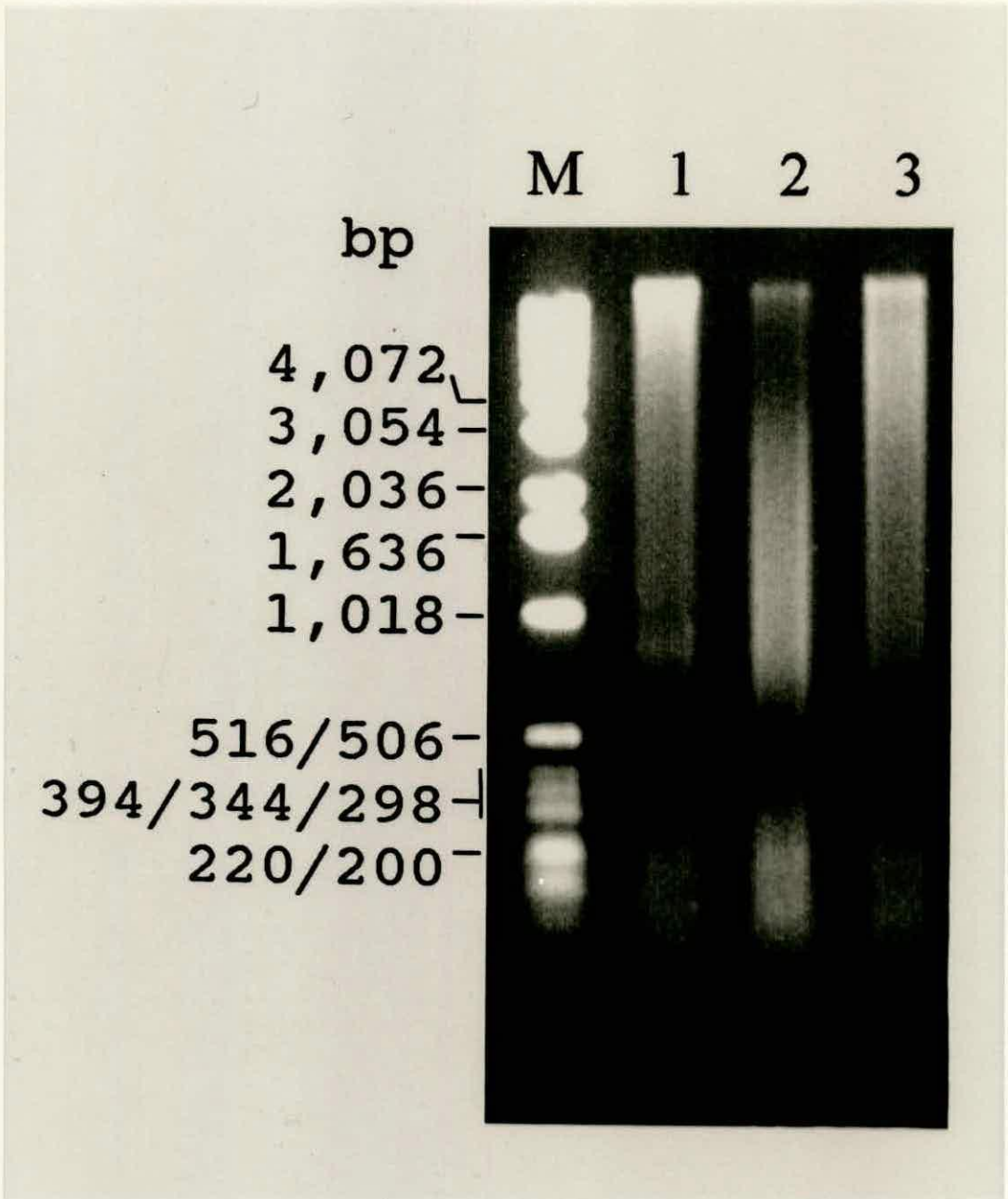

M
1
2
3
bp
4,072
3,054
2,036
1,636
1,018
516/506
394/344/298
220/200

Figure 6.4

Northern blot analysis of KM19.

A Northern blot of adult *T. vitrinus* mRNA (2 μg/track) was hybridised with ^{32}P-labelled KM19 cDNA derived probe.

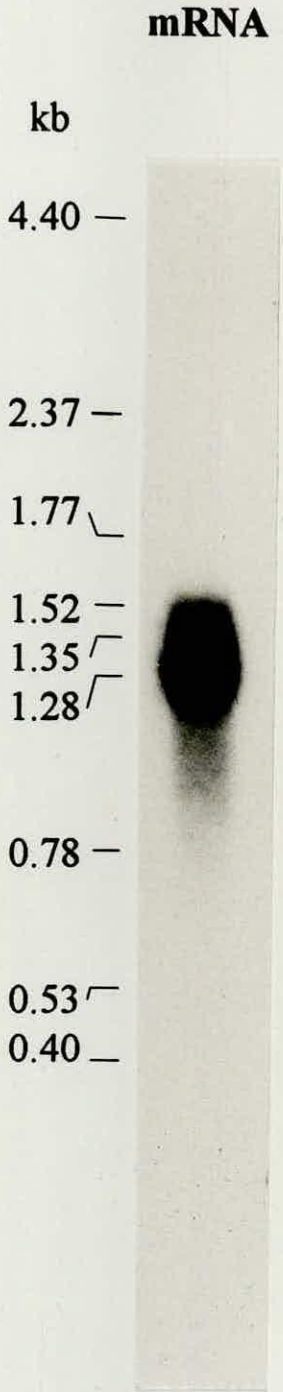

mRNA
kb
4.40
2.37
1.77
1.52
1.35
1.28
0.78
0.53
0.40

Figure 6.5

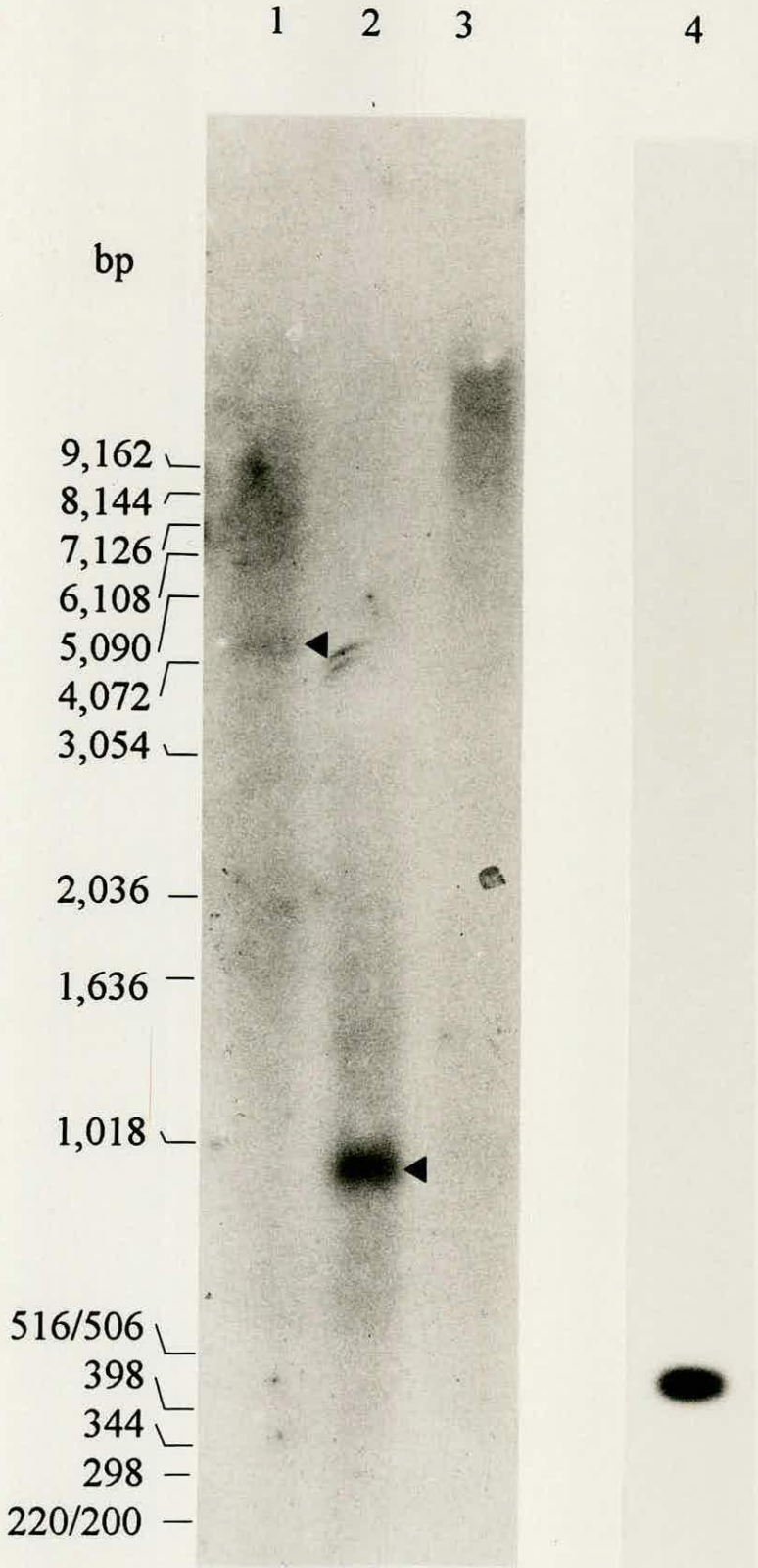
Genomic Southern blot analysis of KM19

A Southern blot of digested adult *T. vitrinus* genomic DNA (2-5 μg/track) was hybridised with a ^{32}P-labelled KM19 cDNA derived probe. The purified 400 bp insert from KM19 was also Southern blotted and hybridised with the probe to act as a positive control.

Lanes:

*Hind*III, *Eco*RI and *Bam*HI digested *T. vitrinus* genomic DNA are shown in lanes 1, 2 and 3 respectively.

4 - KM19 purified insert (DNA template for the probe)


1
2
3
4
bp
9,162
8,144
7,126
6,108
5,090
4,072
3,054
2,036
1,636
1,018
516/506
398
344
298
220/200

6.2.3 PCR amplification of the 5' end of the KM19 cDNA insert

The 5' end sequence for the cDNA encoding the KM19 sequence was sought by PCR amplification using the SL1 primer (table 2.2) and the KM19 specific primers, H0664 (shown in figure 6.1). By comparison of the KM19 serpin-like peptide to several previously sequenced serpins, it was predicted that the length of the sequence from the 5' end of the KM19 cDNA to primer H0664 would be 800-850 bp. Similarly, the expected size from the primer region H0663 to the 3' end of the cDNA is 500-840 bp (see table 6.1). This predicted fragment size would indicate that the full-length cDNA, of which KM19 is part, would be in the region of 1,300 to 1,700 bp in accord with the mRNA transcript size (1,200 bp) indicated by Northern blot analysis using KM19 as a probe (figure 6.4). Primers, M0627 and M0628, which are also shown in figure 6.1, were synthesised at a later date (see section 6.2.7).

Adult *T. vitrinus* (14 days p.i.) cDNA was prepared from mRNA (section 2.11.6). This cDNA was used as the template DNA for PCR amplifications (section 2.11.7) using the oligonucleotide primers SL1 (table 2.2) and H0664 (figure 6.1). The latter primer is directed towards the antisense strand of the KM19 nucleotide sequence (see figure 6.1). An amplification using the KM19 internal primers H0633 and H0664 (figure 6.1) was also carried out. The annealing temperature for the PCR was 60°C and the resultant amplification products were analysed by agarose (0.8%, w/v) gel electrophoresis (section 2.11.8; figure 6.6).

PCR of the cDNA using primers SL1 and H0664 (reaction 1), resulted in the amplification of three strong DNA bands of approx. 740, 540 and 500 bp respectively, slightly shorter than the predicted size (table 6.1). A faint band of 1,170 bp was also present (figure 6.6, lane 1). Amplification, using the internal KM19 primers, H0663 and H0664 (reaction 2), produced a band of approx. 230 bp, which approximates with the predicted size (figure 6.1 and figure 6.6, lane 2).

Table 6.1

Expected size of PCR amplified cDNA products using 5', 3' and KM19 directed primers

				expected size of amplified cDNA fragment(s), based on	
serpin	**total length of serpin** amino acid (bp)	**amino acid identity to KM19**	**amino acids in serpin that KM19 matches**	**length from serpin 5' end to primer H0664 region bp**	**length from primer H0663 region to serpin 3' end bp**
human leukocyte elastase inhibitor (Remold-O'Donnell, Chin and Alberts, 1992)	379 (1137)	33.3% (57.8%)	82-185	843	525
silk moth antitrypsin precursor (Takagi *et al.*, 1990)	392 (1176)	31.8% (54.2%)	102-209	828	624
silk moth antichymotrypsin precursor (Sasaki, 1991)	375 (1125)	31.8% (54.2%)	85-193	846	582
mouse antithrombin III precursor (Wu, Sheffield and Blajchman, 1992)	465 (1395)	34.7%	67-276	843	828
rat serine proteinase inhibitor 3 (SPI 3) precursor (Pages *et al.*, 1990)	408 (1224)	28.9% (54.8%)	119-222	822	669

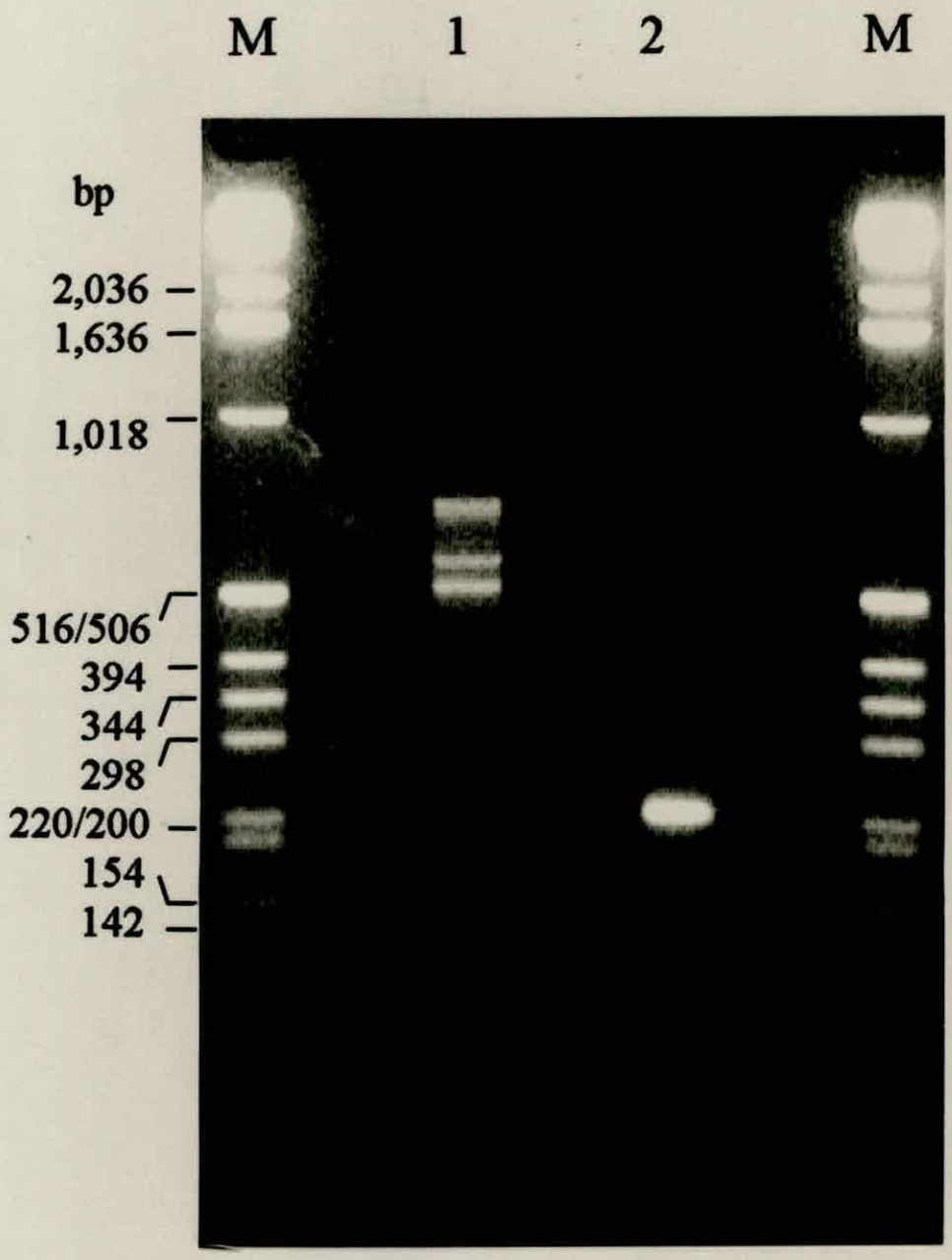
Figure 6.6

Agarose gel profile PCR products amplified from adult *T. vitrinus* cDNA, using KM19 primers.

Adult *T. vitrinus* cDNA was used as the template for PCR reactions, using primer pairs SL1/H0664 and H0663/H0664. The annealing temperature for the reactions was 60°C. The other amplification conditions are stated in section 2.11.7. The amplification products were analysed by agarose (0.8%, w/v) gel electrophoresis.

Lanes:

M - DNA markers
1 - PCR amplification, using primers SL1 and H0664 (reaction 1)
2 - PCR amplification, using primers H0663 and H0664 (reaction 2)


M
1
2
M
bp
2,036
1,636
1,018
516/506
394
344
298
220/200
154
142

6.2.4 Subcloning of the PCR amplification products

The PCR reactions, 1 and 2, from section 6.2.3, were each used as the starting DNA material in ligation reactions with the plasmid vector pCR II (see section 2.10.6). Following transformation, 20 white recombinant colonies from each reaction were selected and the plasmid DNA was extracted from each (section 2.11.14). The plasmid DNA was then digested with *Eco*RI (section 2.11.13) to excise the recombinant insert. The three dominant cDNA bands of 740, 540 and 500 bp in reaction 1 and the 230 bp cDNA in reaction 2 were successfully subcloned into pCR II. The agarose (0.8%, w/v) gel profile of an *Eco*RI restricted recombinant clone for each cDNA is shown in figure 6.7. The excised 230 bp fragment (lane 1) is not visible in the figure, although, it was faintly visible on the actual gel.

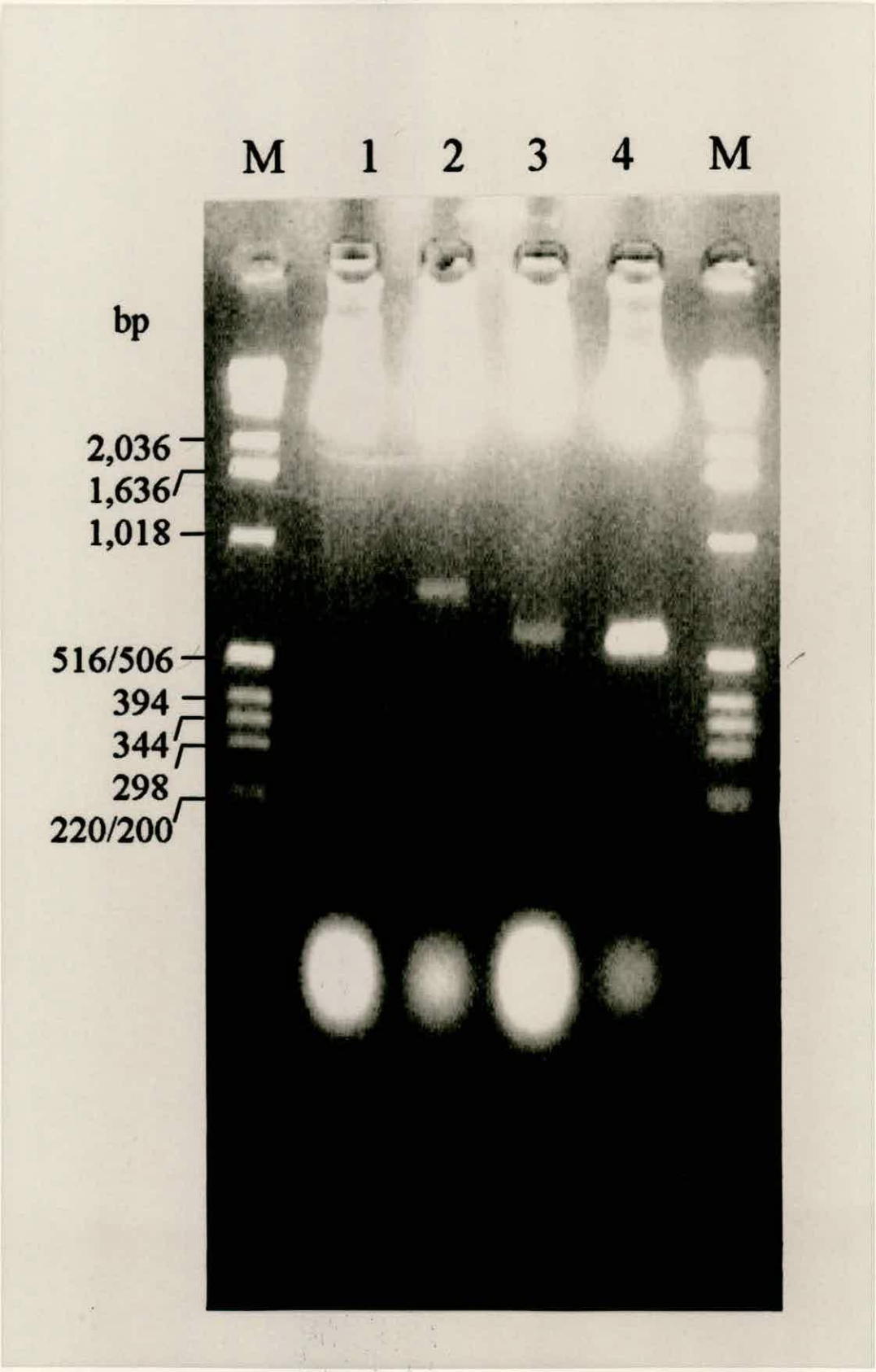
Figure 6.7

Agarose gel profile of *Eco*RI restricted plasmid DNA from recombinant clones.

The products of PCR reactions 1 and 2 were ligated into the pCR II plasmid vector. The plasmid DNA from recombinant clones was isolated and was digested with *Eco*RI to excise their inserts. The digests were analysed by agarose (0.8%, w/v) gel electrophoresis.

Lanes:

M - DNA markers
1 - pCR II plasmid DNA harbouring 230 bp fragment from reaction 2
2 - pCR II plasmid DNA harbouring 740 bp fragment from reaction 1
3 - pCR II plasmid DNA harbouring 540 bp fragment from reaction 1
4 - pCR II plasmid DNA harbouring 500 bp fragment from reaction 1


M
1
2
3
4
M
bp
2,036
1,636
1,018
516/506
394
344
298
220/200

6.2.5 Southern blot analysis of the cDNAs putatively encoding the 5' end of KM19

To confirm that the subcloned PCR fragments described above (figure 6.7) did contain KM19 related sequence an aliquot of PCR reaction 1 from section 6.2.3 and the four *EcoRI* digested recombinant plasmid DNAs from section 6.2.4 were electrophoresed and Southern blotted onto nylon membrane (section 2.11.16). The KM19 insert was PCR amplified and purified, as described in the previous section. An aliquot of the purified KM19 insert was also electrophoresed and Southern blotted (section 2.11.16).

As a probe for Southern blot analysis, the purified KM19 insert was ECL random prime labelled, as described in section 2.11.18. Following hybridisation with KM19, the membranes were washed at 60°C with 0.1% SSC, containing 1% SDS, before detection of the hybridisation signal (section 2.11.18).

The 740, 540 and 500 bp amplified fragments were all recognised by KM19 (figure 6.8) and these fragments were termed serp1, serp2 and serp3 respectively. An additional signal was evident at approx. 800 bp, though in the analysis by agarose gel electrophoresis (figure 6.6) and DNA-PAGE (figure not shown) of the original PCR reaction, a band of this size was not observed. The faint band of 1,170 bp that appeared in the PCR reaction products (figure 6.6) did not hybridise to KM19.

Southern blot analysis of the plasmid DNA from the recombinant pCR II clones confirmed that the three serp fragments were recognised by KM19 (figure 6.9, lanes 1,2 and 3). The subcloned 230 bp fragments that was derived from amplification of the KM19 internal primers H0663 and H0664 (figure 6.9, lane 4), and also the purified KM19 insert (figure 6.9, lane 5) both hybridised with KM19, as would be expected.

Figure 6.8

Southern blot analysis of the PCR products from reaction 1.

An aliquot (10 μl) of PCR reaction 1 (from section 6.2.3) was separated through an agarose (1.2%, w/v) gel before being Southern blotted onto nylon membrane. The blot was probed with ECL random prime labelled KM19 cDNA insert. A final stringency wash in 0.1% (v/v) SSC, containing 0.1% (w/v) SDS at 60°C was performed before a hybridisation signal was sought.

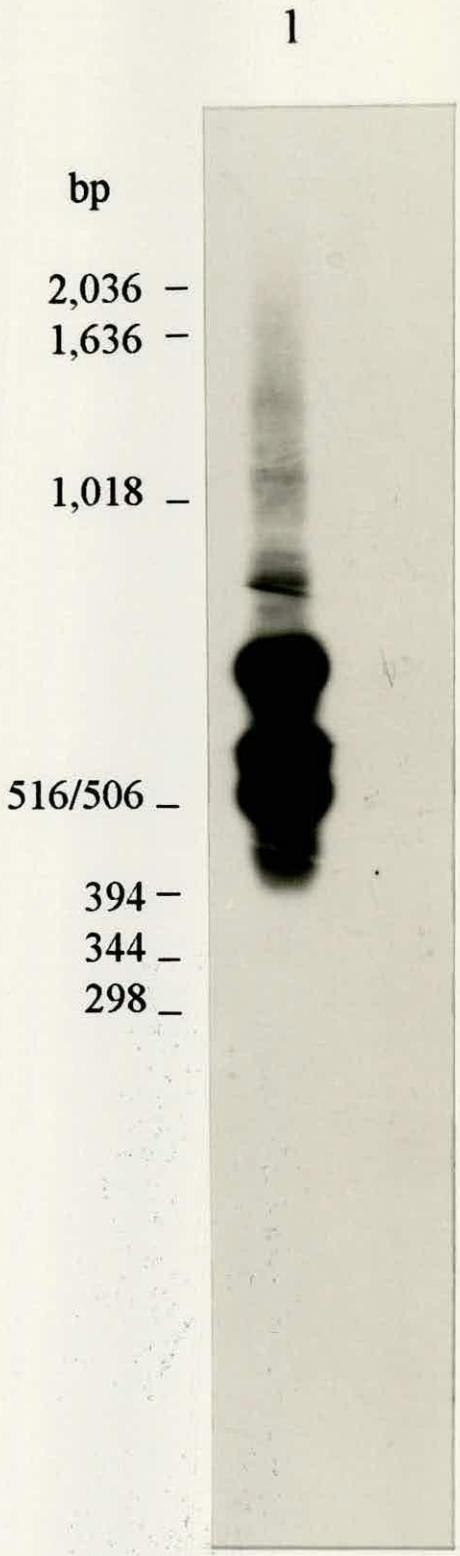

1
bp
2,036
1,636
1,018
516/506
394
344
298

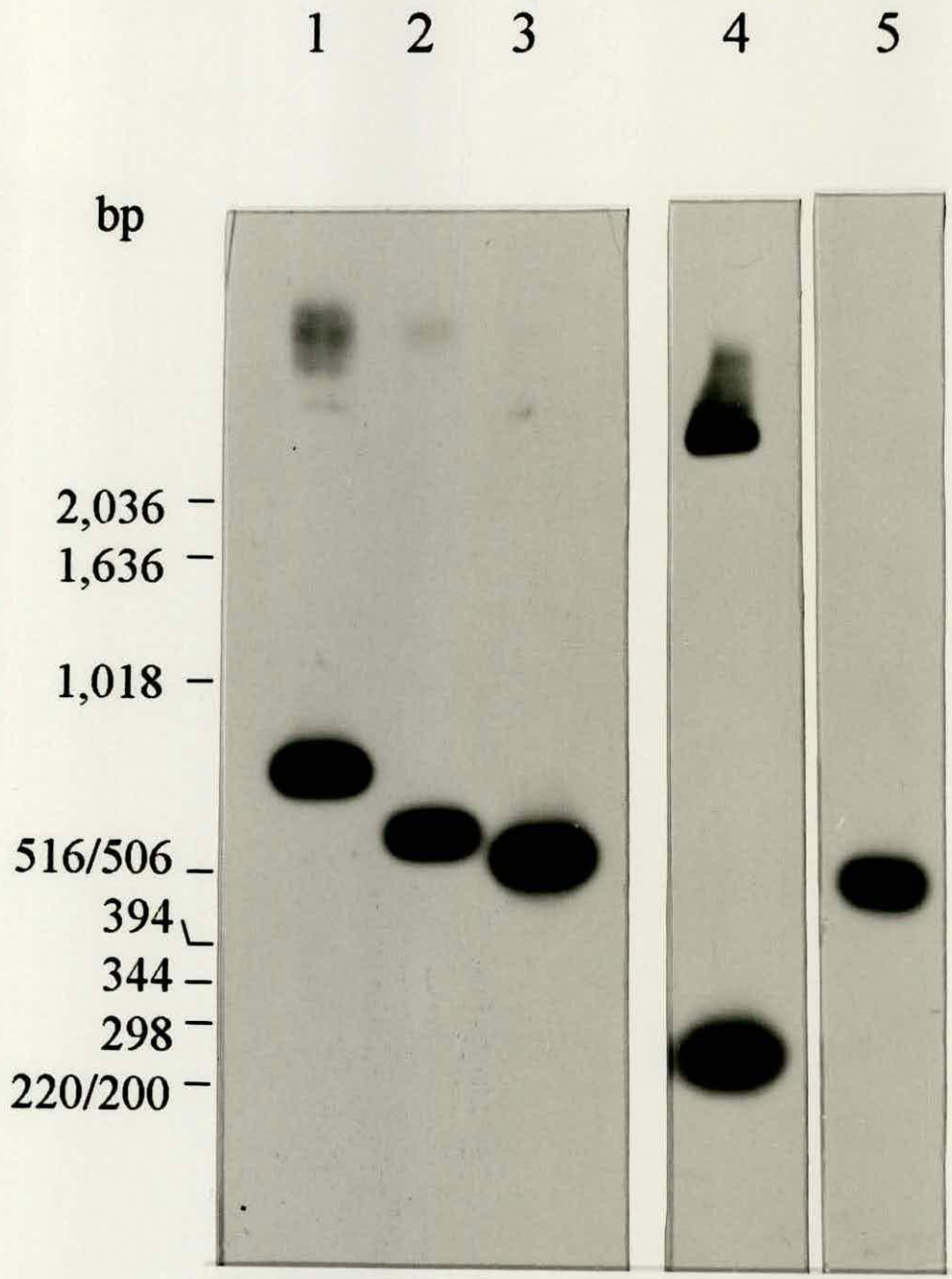
Figure 6.9

Southern blot analysis of subcloned PCR products from reactions 1 and 2.

The PCR products from reactions 1 and 2, section 6.2.3, were subcloned into the pCR II plasmid vector. The plasmid DNA from the recombinant clones was extracted and digested with *Eco*RI to excise the DNA inserts. The products of digestion were fractionated in an agarose (0.8%, w/v) gel and were Southern blotted onto a nylon membrane. The blots were probed with ECL random prime labelled KM19 cDNA insert. A final stringency wash in 0.1% SSC, containing 0.1% SDS at 60°C was performed before a hybridisation signal was sought.

Lanes:

1 - pCR II plasmid DNA harbouring 740 bp fragment from reaction 1
2 - pCR II plasmid DNA harbouring 540 bp fragment from reaction 1
3 - pCR II plasmid DNA harbouring 500 bp fragment from reaction 1
4 - pCR II plasmid DNA harbouring 230 bp fragment from reaction 2
5 - KM19 400 bp purified insert (template DNA for the probe)


1
2
3
4
5
bp
2,036
1,636
1,018
516/506
394
344
298
220/200

6.2.6 Sequence analysis of the cDNAs putatively encoding the 5' end of KM19

The subcloned serp PCR fragments from reaction 1 and the 230 bp fragment from reaction 2 in section 6.2.3 were sequenced three times by di-deoxy chain termination (section 2.11.15), using M13 forward and reverse sequencing primers (see table 2.2). The larger serp1 fragment was also sequenced using primers SL1 and H0664.

The actual size of the serp cDNA fragments were 728, 584 and 528 bp respectively and each was compared with the nucleotide sequence of KM19 (figure 6.10). The KM19 sequence from bp 82-353 aligned exactly with the last 272 bp of serp1, serp2 and serp3, apart from the fact that serp1 contained an adenosine instead of a cytidine at position 565 of the serp1 sequence. The first 81 bp of KM19 was not homologous to the corresponding regions of any of the other fragments. The fragment which was originally derived from PCR using KM19 primers, H0663 and H0664, was 227 nucleotides in length and corresponded exactly to 127-353 bp of KM19, as expected.

The first 22 bp of each of the serp fragments corresponded to the SL1 sequence. The serp1 sequence from bases 167-728 was almost identical to bases 23-584 of serp2. Similarly, serp1 bases 222-728 and serp2, bases 78-584, aligned with serp3, bases 23-528. The only differences were as follows: base 26 of serp2 contained a thymidine, whereas the equivalent position in serp1 (base 170) harboured an adenosine; at position 40 of serp3, there was a guanosine, instead of an adenosine; serp1 contained an adenosine and cytidine at nucleotides 376 and 450 respectively, compared to the guanosine and adenosine at the equivalent positions in serp2 and serp3.

Following the SL1 sequence in serp3, translation of the nucleotide sequence from nucleotide 24 gave a reading frame of 168 amino acids straight-through. Translation of serp2 from nucleotide 25 and serp1 from nucleotide 109 also produced reading frames straight-through. In the other two possible open reading frames for the fragments, translation produced stop codons throughout the peptide sequences. The first codon in serp3 was a Met, therefore, this may be a putative start codon. Serp1 and serp2 also contained a Met at the position corresponding to the serp3 Met,

although both serp1 and serp2 also had a Met seven amino acids upstream from this. The serp peptides and the KM19 serpin-like peptide were aligned with human leucocyte elastase (LEI; Remold-O'Donnell, Chin and Alberts, 1992), human placental thrombin inhibitor (HPTI; Coughlin *et al.*, 1993), rat serine proteinase inhibitor-3 (SPI3; Pages *et al.*, 1990) and murine kallikrein-binding protein (KBP; Chai, Chao and Chao, 1991) [figure 6.11]. The KM19 serpin-like peptide was 107 amino acids in length and each serp fragment showed 100% identity with KM19 over 91 amino acids, apart from serp1 which had a Met at amino acid 153, instead of a Leu. The serp peptides, commencing from the consensus putative start codon, showed 33.2%, 31.2%, 28.9% and 28.3% identity with HPTI, LEI, SPI3 and KBP respectively.

Figure 6.10

Alignment of the nucleotide sequences for KM19, serp1, serp2 and serp3.

```
serp1  GGTTTAATTA CCCAAGTTTG AGTGTCTGCA TAAATTAGCG GAAGCTCTCC  50
serp2  .......... .......... .......... .......... ..........
serp3  .......... .......... .......... .......... ..........
 KM19  .......... .......... .......... .......... ..........

serp1  GAATGTGAAG AAACAGACCA CAGAGAAAAA TCTCACTGAA GTGGAAGGAG 100
serp2  .......... .......... .......... .......... ..........
serp3  .......... .......... .......... .......... ..........
 KM19  .......... .......... .......... .......... ..........

serp1  AACAGTGAAT ATCTCTTTGG CCAGTTGTGA AAGATATCCT TGGATTTTTA 150
serp2  .......... .......... .......... .......... ....GGTTTA   6
serp3  .......... .......... .......... .......... ..........
 KM19  .......... .......... .......... .......... ..........

serp1  AATGGTAACA GAAAAGAGCA TCGCTCCTAT TCCACCTTGT TCCACGCCGT 200
serp2  ATTACCCAAG TTTGAGAGCT TCGCTCCTAT TCCACCTTGT TCCACGCCGT  56
serp3  .......... .......... .......... .......... .........G   1
 KM19  .......... .......... .......... .......... ..........

serp1  AATGTCTGAC GCGGTGGAAA CAATGTTTTT AACGGCGGAA ACGGACTTTG 250
serp2  AATGTCTGAC GCGGTGGAAA CAATGTTTTT AACGGCGGAA ACGGACTTTG 106
serp3  GTTTAATTAC CCAAGTTTGA GAATGTTTTT AACGGCGGGA ACGGACTTTG  51
 KM19  .......... .......... .......... .......... ..........

serp1  GGCTGAATAT GCTGAAGCAT GCTCCTGCCA ACGAATCCTT GGTCGTGTCG 300
serp2  GGCTGAATAT GCTGAAGCAT GCTCCTGCCA ACGAATCCTT GGTCGTGTCG 156
serp3  GGCTGAATAT GCTGAAGCAT GCTCCTGCCA ACGAATCCTT GGTCGTGTCG 101
 KM19  .......... .......... .......... .......... ..........

serp1  CCACTCTCTG TCATATTCGC TTTGGCCATG GTTCAGGCAG GAGCAAAAGG 350
serp2  CCACTCTCTG TCATATTCGC TTTGGCCATG GTTCAGGCAG GAGCAAAAGG 206
serp3  CCACTCTCTG TCATATTCGC TTTGGCCATG GTTCAGGCAG GAGCAAAAGG 151
 KM19  .......... .......... .......... .......... ..........

serp1  AACCACAAAA TCTCAGATCA GTGCTATCCT CTCCAAAGGA TCGTCTGACA 400
serp2  AACCACAAAA TCTCAGATCA GTGCTGTCCT CTCCAAAGGA TCGTCTGACA 256
serp3  AACCACAAAA TCTCAGATCA GTGCTGTCCT CTCCAAAGGA TCGTCTGACA 201
 KM19  .......... .......... ....GAAGCA GAATAGCTAA TGGATTTTTC  26
```

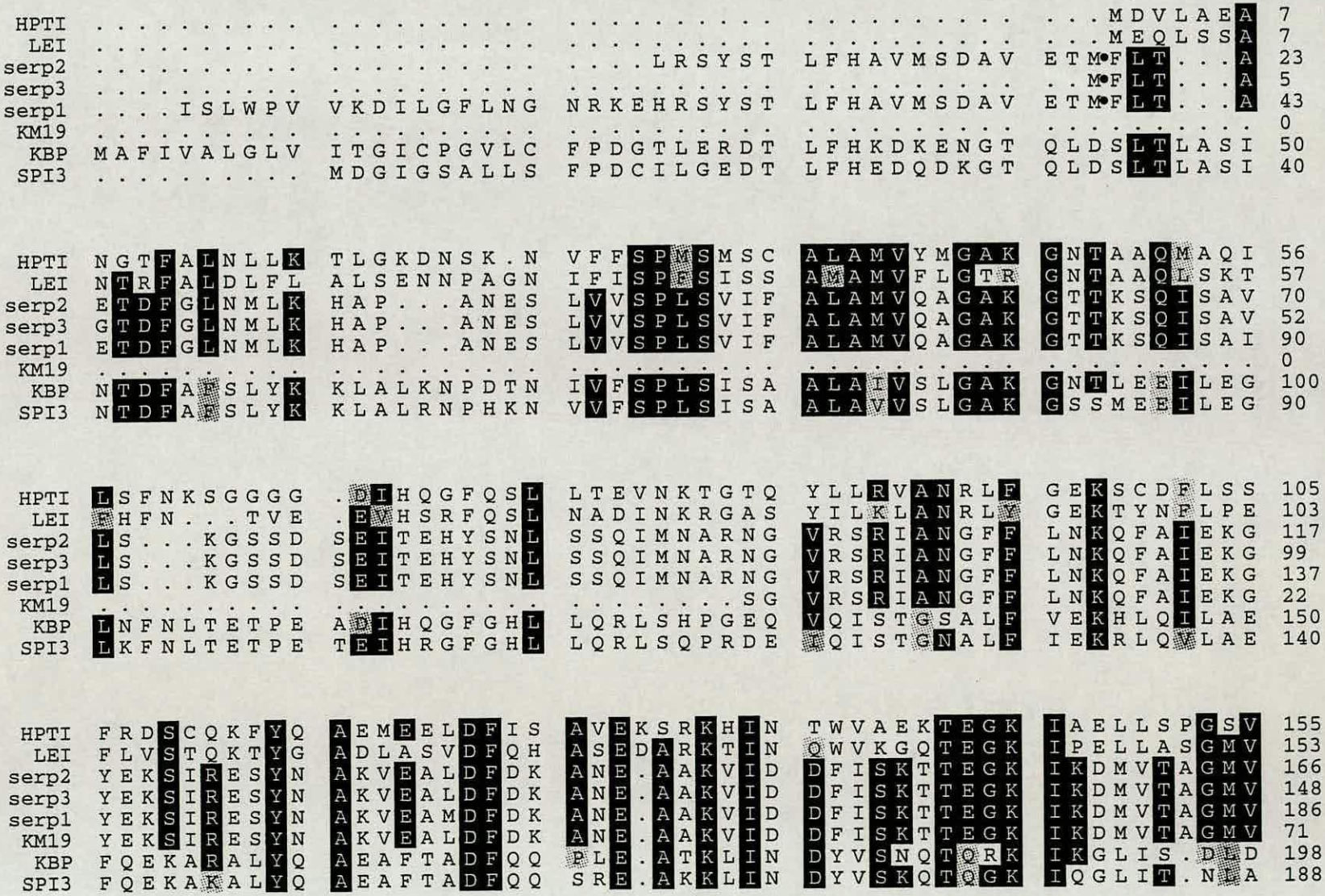
```
serp1  GCGAAATCAC GGAGCACTAC TCTAATCTTT CCAGCCAGAT CATGAACGCC 450
serp2  GCGAAATCAC GGAGCACTAC TCTAATCTTT CCAGCCAGAT CATGAACGCA 301
serp3  GCGAAATCAC GGAGCACTAC TCTAATCTTT CCAGCCAGAT CATGAACGCA 255
 KM19  CTGAACAAAC AGTTCGCAAT TCTGCTTCTA ACA.CCGTTT CGTGCGTTCA  75

serp1  CGAAACGGTG TTAGAAGCAG AATAGCTAAT GGATTTTTCC TGAACAAACA 500
serp2  CGAAACGGTG TTAGAAGCAG AATAGCTAAT GGATTTTTCC TGAACAAACA 356
serp3  CGAAACGGTG TTAGAAGCAG AATAGCTAAT GGATTTTTCC TGAACAAACA 301
 KM19  TGATCTGGTG TTAGAAGCAG AATAGCTAAT GGATTTTTCC TGAACAAACA 125

serp1  GTTCGCAATT GAAAAAGGCT ATGAGAAATC TATCAGAGAA AGCTACAATG 550
serp2  GTTCGCAATT GAAAAAGGCT ATGAGAAATC TATCAGAGAA AGCTACAATG 406
serp3  GTTCGCAATT GAAAAAGGCT ATGAGAAATC TATCAGAGAA AGCTACAATG 351
 KM19  GTTCGCAATT GAAAAAGGCT ATGAGAAATC TATCAGAGAA AGCTACAATG 175

serp1  CGAAAGTGGA AGCTATGGAT TTTGACAAAG CAAACGAAGC TGCAAAGGTT 600
serp2  CGAAAGTGGA AGCTCTGGAT TTTGACAAAG CAAACGAAGC TGCAAAGGTT 456
serp3  CGAAAGTGGA AGCTCTGGAT TTTGACAAAG CAAACGAAGC TGCAAAGGTT 401
 KM19  CGAAAGTGGA AGCTCTGGAT TTTGACAAAG CAAACGAAGC TGCAAAGGTT 225

serp1  ATCGATGATT TTATAAGCAA GACGACTGAG GGGAAAATCA AGGACATGGT 650
serp2  ATCGATGATT TTATAAGCAA GACGACTGAG GGGAAAATCA AGGACATGGT 506
serp3  ATCGATGATT TTATAAGCAA GACGACTGAG GGGAAAATCA AGGACATGGT 451
 KM19  ATCGATGATT TTATAAGCAA GACGACTGAG GGGAAAATCA AGGACATGGT 275

serp1  GACAGCAGGC ATGGTTAAAG ATGCTTACTC CCTTATTGTC AATGCCATCT 700
serp2  GACAGCAGGC ATGGTTAAAG ATGCTTACTC CCTTATTGTC AATGCCATCT 556
serp3  GACAGCAGGC ATGGTTAAAG ATGCTTACTC CCTTATTGTC AATGCCATCT 501
 KM19  GACAGCAGGC ATGGTTAAAG ATGCTTACTC CCTTATTGTC AATGCCATCT 325

serp1  ATTTCACTGC TGAATGGGTG GAAAAGTT.. ..........  .......... 728
serp2  ATTTCACTGC TGAATGGGTG GAAAAGTT.. ..........  .......... 584
serp3  ATTTCACTGC TGAATGGGTG GAAAAGTT.. ..........  .......... 528
 KM19  ATTTCACTGC TGAATGGGTG GAAAAGTTCT ACAAAAGTTC CAATTCAAAC 375

serp1  .......... .......... ...
serp2  .......... .......... ...
serp3  .......... .......... ...
 KM19  GAGACATTTT ATAGCACAGC AGC 398
```

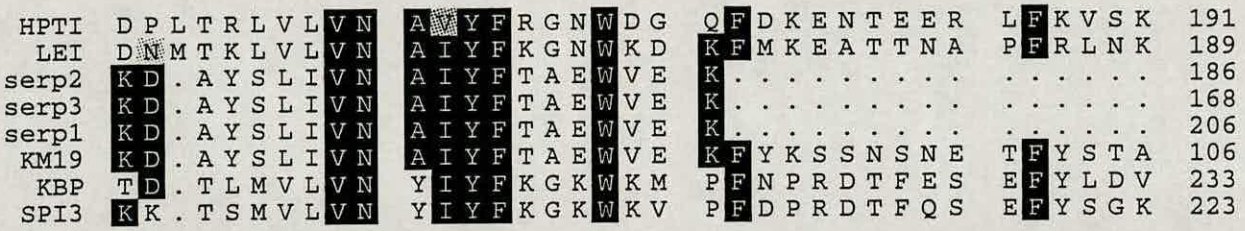
The nucleotide sequences for KM19, serp1, serp2 and serp3 were aligned. The SL1 primer sequence in each of the serp nucleotide sequences is underlined. The 81 bp of KM19 which does not match the corresponding region in the serp sequences is double underlined. There are five single base changes in the overlapping regions (▲). Serps 1, 2 and 3 possessed a consensus putative start codon (ATG, marked ■).

Figure 6.11

Alignment of the derived amino acid sequences for KM19, serp1, serp2 and serp3 with the peptide sequences for four serpins.

The deduced amino acid sequences for serpin-like peptide of KM19 (see figure 2), serp1, serp2 and serp3 were aligned with similar regions in the peptide sequences for human LEI (Remold-O'Donnell, Chin and Alberts, 1992), rat SPI-3 (Pages *et al.*, 1990), murine KBP (Chai, Chao and Chao, 1991) and HPTI (Coughlin *et al.*, 1993). The consensus putative start Met for serps 1, 2 and 3 is marked (M●). Identical amino acids (■) and highly conserved (▩) and conserved (▒) amino acids between the eight sequences are shown. The full length peptide sequences for LEI, SPI-3, KBP and HPTI are 379, 408, 417 and 376 respectively.


HPTI . . .


HPTI D P L T R L V L V N A V Y F R G N W D G Q F D K E N T E E R L F K V S K 191
LEI D N M T K L V L V N A I Y F K G N W K D K F M K E A T T N A P F R L N K 189
serp2 K D . A Y S L I V N A I Y F T A E W V E K 186
serp3 K D . A Y S L I V N A I Y F T A E W V E K 168
serp1 K D . A Y S L I V N A I Y F T A E W V E K 206
KM19 K D . A Y S L I V N A I Y F T A E W V E K F Y K S S N S N E T F Y S T A 106
KBP T D . T L M V L V N Y I Y F K G K W K M P F N P R D T F E S E F Y L D V 233
SPI3 K K . T S M V L V N Y I Y F K G K W K V P F D P R D T F Q S E F Y S G K 223

6.2.7 PCR amplification using KM19 specific primers

(a) Design of primers unique to KM19

Amplification of adult *T. vitrinus* cDNA using PCR and the primers SL1 and H0664 (KM19 specific), resulted in the amplification of three cDNA fragments that were very similar to the last 317 nucleotides of the KM19 insert sequence but did not contain sequence that corresponded to the first 81 bp of KM19 (section 6.2.3 to 6.2.6). Following this, additional primers were designed that were unique to KM19. As the first 47 bp of KM19 was repeated further downstream in KM19 and serps 1, 2 and 3, KM19 specific primers could only be directed towards the sequence from bases 48-81. The chosen primers, M0627 (sense) and M0628 (antisense), are detailed in figure 6.1 and were directed towards nucleotides 48 to 70. Amplification from M0627 to the 3' end of the cDNA should include 11 bases (bp 71-81) which are unique to KM19 and so confirm that the amplified cDNA is derived from KM19. However, 14 bp of the 5' end of the sense primer M0627 (bp 48-61 of KM19) was found to occur in the reverse complement on KM19 from bp 95-82 and therefore may also act as an antisense primer.

(b) PCR reactions using adult *T. vitrinus* cDNA

Adult *T. vitrinus* (14 days p.i.) cDNA was prepared (section 2.11.6) and used as the template DNA for the PCR reactions (section 2.11.7). Initially, attempts were made to specifically amplify the putative 5' end of KM19 and so the following primer pairs were chosen, each of which includes a KM19 specific primer: SL1/M0628 (reaction 1), SL1/M0627 (reaction 2), M0627/H0664 (reaction 3), M0628/H0664 (reaction 4) and H0663/H0664 (reaction 5). In each case, the first primer was the sense primer. The annealing temperature applied to the PCR was 55°C and the products of the PCR reactions were analysed by agarose (0.8%, w/v) gel electrophoresis (section 2.11.8; figure 6.12).

Amplification using primers SL1 and M0628 produced a single cDNA band of approx. 350 bp (figure 6.12, lane 1). The primer pair, SL1/M0627, produced a smear of DNA across the tract, a profile that is indicative of a single primer annealing (figure 6.12, lane 2). PCR using primers M0627 to H0664 (figure 6.12, lane 3) and

H0663 to H0664 (figure 6.12, lane 5) gave products of approx. 300 and 230 bp respectively, as expected. Amplification of M0628 to H0664 (figure 6.12, lane 4) resulted in a clear single band of approx. 290 bp, with smearing of DNA at a higher molecular weight.

The 350 bp cDNA fragment generated in reaction 1 is at the lower end of the predicted size range, as based on the serp sequences. The remainder of this PCR reaction was electrophoresed through an agarose (0.8%, w/v) gel (section 2.11.8), the 350 bp band was excised and was purified by gencleaning (section 2.11.9). Following this, the DNA was extracted with phenol:chloroform extracted (section 2.11.1) and precipitated in ethanol (section 2.11.2). The DNA from this preparation was then ligated into the pCR II vector (section 2.10.6). Several recombinant colonies were obtained from which the plasmid DNA was extracted and digested with *Eco*RI (section 2.11.14). Analysis of the excised inserts revealed that five different sized cDNAs, varying from 300-350 bp, had been subcloned (results not shown). Each of these cDNAs were sequenced using the di-deoxy termination reaction and the M13 forward and reverse primers (section 2.11.15). The derived nucleotide sequences from all of the five cDNAs were found to be primed at each end with the M0628 primer and none of the cDNA sequences matched KM19 (results not shown). The 300 bp fragment obtained in reaction 3 was also subcloned and sequenced, as described above. The nucleotide sequence of this fragment was found to be identical to the KM19 sequence from bases 48 to 353 (results not shown).

Amplifications were also carried out using primer pairs M0627/dT, M0628/dT, and H0663/dT. As the dT primer, which is directed to the poly A+ tail of mRNA has a low melting temperature, the PCR reactions were carried out at 38°C. The products from both amplifications involving primers M0627/dT and H0663/dT gave a smear of DNA across the tract, suggesting only one primer in each reaction was annealing to the template. No amplification products were obtained using primers M0628 and dT (results not shown).

(c) PCR reactions using amplified adult *T. vitrinus* cDNA λgt11 library

From the above results, it appeared that the full length KM19 cDNA may not contain the SL1 primer site at the 5' end. Also, there may be difficulty in amplifying the 3' end using the dT primer. This primer requires a low annealing temperature which leads to a decreased specificity of the second primer. It was decided, therefore, to perform amplifications of the amplified adult *T. vitrinus* cDNA λgt11 library (see section 2.10.12) using the KM19 specific primers and the λgt11 primers 514N and 515N (table 2.2). The KM19 cDNA may be in two possible orientations within λgt11 as detailed in the schematic diagram in figure 6.13.

A number of PCR amplifications of the λgt11 library were initially carried out using 514N and 515N in different combinations with the KM19 primers. The PCR products were analysed by agarose (0.8%, w/v) gel electrophoresis and a selection of the results are shown in figure 6.14 (lanes 1-6) and the results are summarised in table 6.2. When the library was amplified using primers 514N and M0628 at an annealing temperature of 55°C (reaction 1), the resultant products comprised a dominant band of approx. 650 bp, with fainter bands of about 400, 320 and 200 bp (figure 6.14, lane 1). In orientation A (see figure 6.13), the 5' end of KM19 would be amplified (240-540 bp in size) while, in orientation B (figure 6.13), the 3' region of KM19 would be amplified (500-850 bp). Therefore, the amplification products observed indicated that both amplifications had taken place. The product of reaction 1 (figure 6.14, lane 1) was then used as the template for further amplifications using primers M0627/polydT (reaction 2) and M0628/polydT (reaction 3) at an annealing temperature of 38°C. Reaction 2 did not clearly amplify any cDNA fragments (figure 6.14, lane 2) but reaction 3 produced a clear single band of approx. 650 bp (figure 6.14, lane 3). Reaction 3 was used as target DNA for a PCR using primers H0663/H0664 (reaction 4), H0663/polydT (reaction 5) and M0628/polydT (reaction 6) at an annealing temperature of 38°C. Reaction 4 generated a single band of 230 bp (figure 6.14, lane 4) and reaction 5 yielded a clear cDNA fragment of 450 bp (figure 6.14, lane 5). The re-amplification of the reaction, using primers M0628 and polydT (reaction 6), produced two fragments of approx. 700 and 400 bp (figure 6.14, lane 6).

To consolidate these results, the adult *T. vitrinus* cDNA λgt11 amplified library was subjected further to PCR, using primer combinations 514N/M0627 (reaction 7), 514N/M0628 (reaction 8), 515N/M0627 (reaction 9) and 515N/M0628 (reaction 10), at a more stringent annealing temperature of 60°C. The PCR products were analysed by agarose (0.8%, w/v) gel electrophoresis (figure 6.14, lanes 7, 8, 9 and 10). Reaction 7, using the 514N/M0627 primer combination, resulted in two strong DNA bands of 380 and 480 bp and several weaker bands ranging from 200 to 980 bp (figure 6.14, lane 7). Reaction 8, with primers 514N/M0628, produced an individual definite cDNA fragment of 650 bp and fainter bands <350 bp (figure 6.14, lane 8). Reaction 9, primers 515N/M0627 amplified a ladder of fragments ranging from <200-1,000 bp (figure 6.14, lane 9). The final amplification, reaction 10, did not give any amplified products (figure 6.14, lane 10).

(d) Subcloning and sequencing of the PCR products

PCR reactions 7, 8 and 9 were used as the templates in ligation reactions with the plasmid vector, pCR II (section 2.10.6). Following transformation, recombinant colonies were isolated, the plasmid DNA from each was restricted with *Eco*RI (section 2.11.13) and analysed by agarose (0.8%, w/v) gel electrophoresis (section 2.11.8). No recombinant colonies were obtained for reaction 9. A selection of the clones are shown in figure 6.15. From reaction 7, two fragments of approx. 500 bp were subcloned (figure 6.15, lanes 3 and 5). From reaction 8, six cDNAs, ranging from 200-500 bp in size (figure 6.15, lanes 6-11) were subcloned. After hybridisation analysis of these recombinants, using KM19 as a probe, five putative positive plasmids were isolated and sequenced by Ms. J. Bartley at the University of Durham, using the M13 forward and reverse primers. On analysis of the derived nucleotide sequences, none of the clones contained sequence corresponding to KM19. Three of the fragments (from lanes 3, 5 and 8) were found to contain different regions of the λgt11 sequence, the other two clones contained sequence that was not homologous to any of the sequences present in the databases.

Figure 6.12

Agarose gel profile of PCR amplifications of adult *T. vitrinus* cDNA using KM19 specific primers.

Adult *T. vitrinus* cDNA was subjected to PCR amplification, using the primer pairs, SL1/M0628, SL1/M0627, M0627/H0664, M0628/H0664 and H0663/H0664, at an annealing temperature of 60°C. The resultant PCR products were analysed by agarose (0.8%, w/v) gel electrophoresis.

Lanes:

1 - PCR amplification, using primers SL1 and M0628 (reaction 1)
2 - PCR amplification, using primers SL1 and M0627 (reaction 2)
3 - PCR amplification, using primers M0627 and H0664 (reaction 3)
4 - PCR amplification, using primers M0628 and H0664 (reaction 4)
5 - PCR amplification, using primers H0663 and H0664 (reaction 5)
M - DNA markers

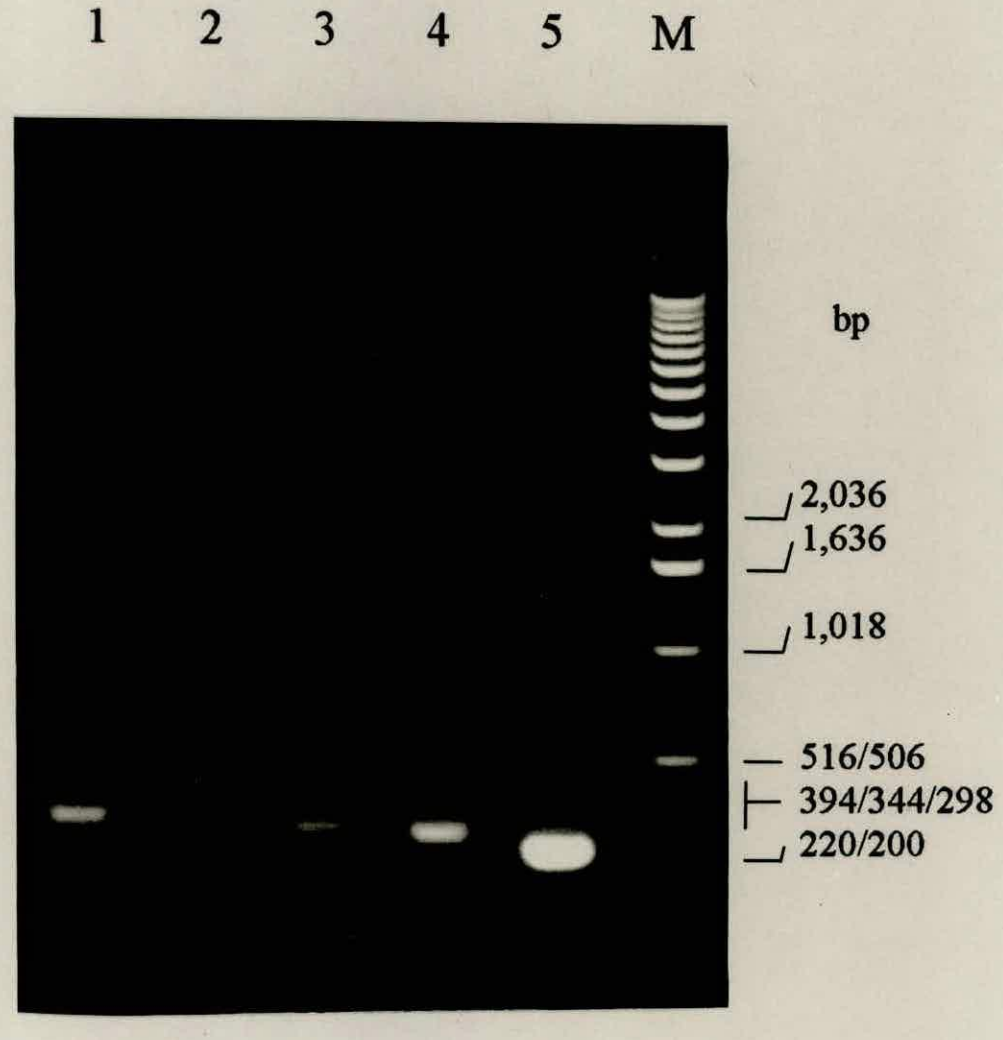
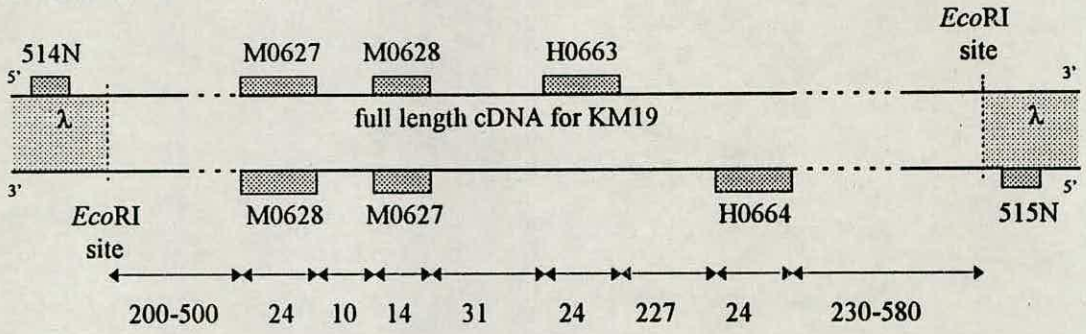

1
2
3
4
5
M
bp
2,036
1,636
1,018
516/506
394/344/298
220/200

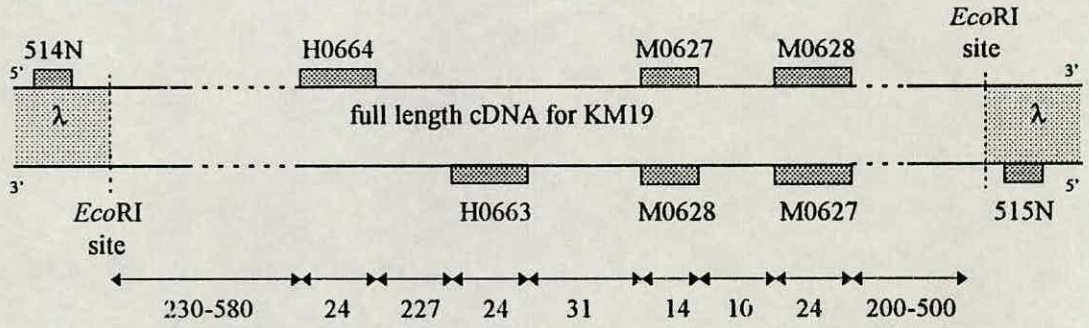
Figure 6.13

Schematic diagram of the KM19 full length cDNA in λgt11.

Orientation A:



Orientation B:



The cDNA may be inserted in two orientations within the λgt11 *Eco*RI cloning site. If the cDNA is in the same orientation as the β-galactosidase gene, the λgt11 primer, 514N, will be situated upstream of the 5' end of the KM19 cDNA sense strand (orientation A). If the cDNA is in the opposite orientation, the 515N primer will be upstream of 5' end of the KM19 cDNA sense strand (orientation B). The regions to which the KM19 primers (see figure 6.10) and λgt11 primers (see table 2.2) are directed are shown (▭). Distance between the primers and the putative 5' and 3' regions of the cDNA are given in bp (⟷).

Table 6.2

Summary of the PCR amplifications of adult *T. vitrinus* cDNA λgt11 library, using λgt11 and KM19 specific primers (section 6.2.7 [c]).

reaction number	primer pairing (sense/ antisense)	target DNA	annealing temp, (°C)	expected fragment size* (bp) if target DNA is in orientation		Observed size, (bp)
				A	B	
1	514N/M0627	λgt11 library	55	240-540	500-850	650 [400, 320,200]
2	M0627/polydT	reaction 1	38	no products	530-890	no products
3	M0628/polydT	reaction 1	38	no products	500-850	650
4	H0663/H0664	reaction 3	38	no products	227	230
5	H0663/polydT	reaction 3	38	no products	450-810	450
6	M0628/polydT	reaction 3	38	no products	500-850	700, 400
7	514N/M0627	λgt11 library	60	240-540	530-890	380, 480 [200-980]
8	514N/M0628	λgt11 library	60	220-520	500-850	650 [<350]
9	515N/M0627	λgt11 library	60	500-850	220-520	200-1,000
10	515N/M0628	λgt11 library	60	540-540	530-890	no products

* Predicted fragment sizes are based table 6.1.

Figure 6.14

Agarose gel profile of PCR amplifications of an adult *T. vitrinus* cDNA λgt11 amplified library, using KM19 specific primers.

Adult *T. vitrinus* cDNA λgt11 amplified library was subjected to PCR using a variety of primers. The annealing temperature was dependent on the primer composition. The resultant PCR products were analysed by agarose (0.8%, w/v) gel electrophoresis.

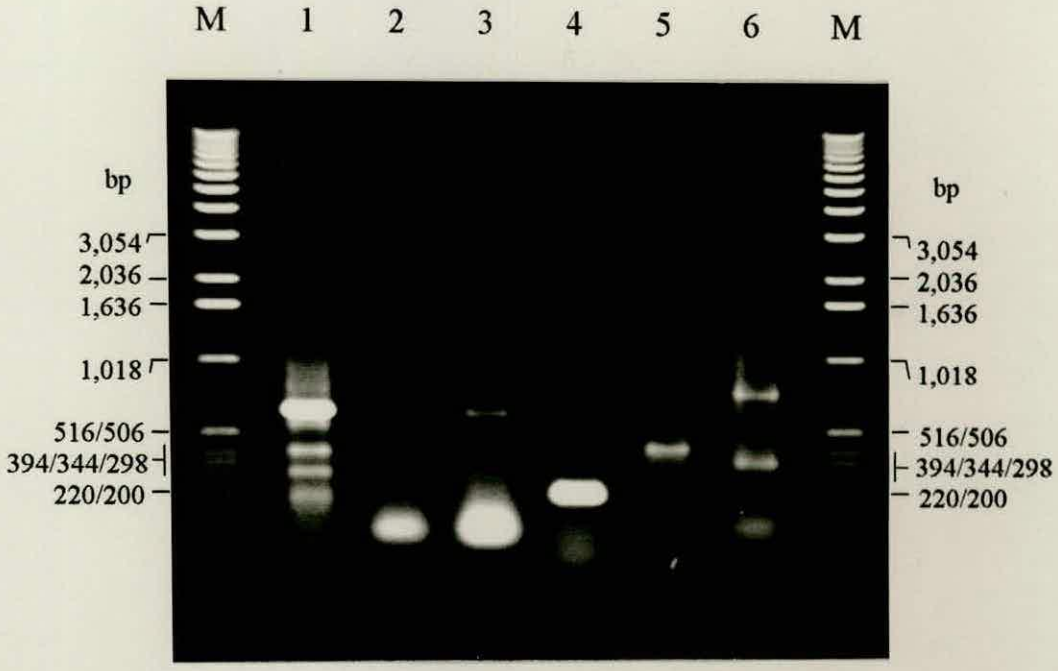
(a)

Lane	template for PCR	primers	annealing temp.	
1 -	cDNA λgt11 library	514N/M0628	55°C	(reaction 1)
2 -	reaction 1	M0628/polydT	38°C	(reaction 2)
3 -	reaction 1	M0628/polydT	38°C	(reaction 3)
4 -	reaction 3	H0663/H0664	38°C	(reaction 4)
5 -	reaction 3	H0663/polydT	38°C	(reaction 5)
6 -	reaction 3	M0628/polydT	38°C	(reaction 6)

(b)

Lane	template for PCR	primers	annealing temp.	
1 -	cDNA λgt11 library	514N/M0627	60°C	(reaction 7)
2 -	cDNA λgt11 library	514N/M0628	60°C	(reaction 8)
3 -	cDNA λgt11 library	515N/M0627	60°C	(reaction 9)
4 -	cDNA λgt11 library	515N/M0628	60°C	(reaction 10)

M - DNA markers


M 1 2 3 4 5 6 M
bp
3,054
2,036
1,636
1,018
516/506
394/344/298
220/200
bp
3,054
2,036
1,636
1,018
516/506
394/344/298
220/200

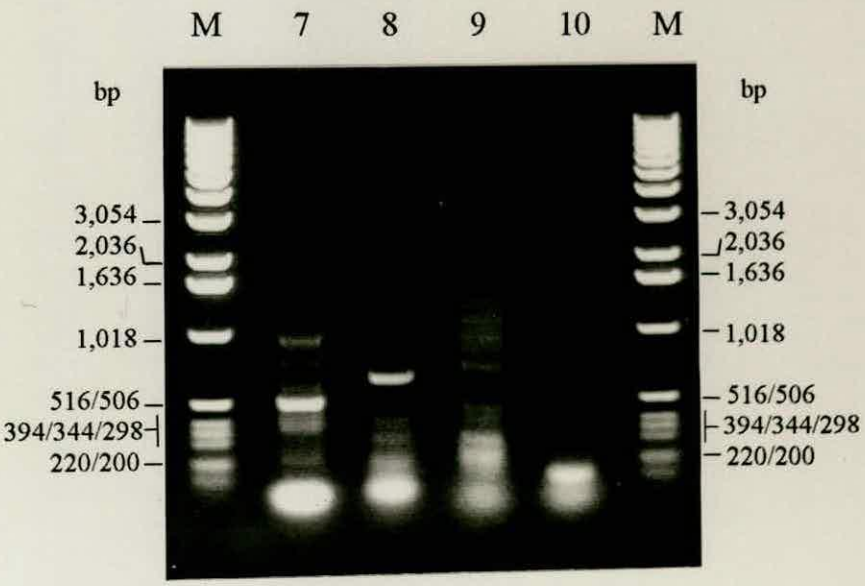

M 7 8 9 10 M
bp
3,054
2,036
1,636
1,018
516/506
394/344/298
220/200
bp
3,054
2,036
1,636
1,018
516/506
394/344/298
220/200

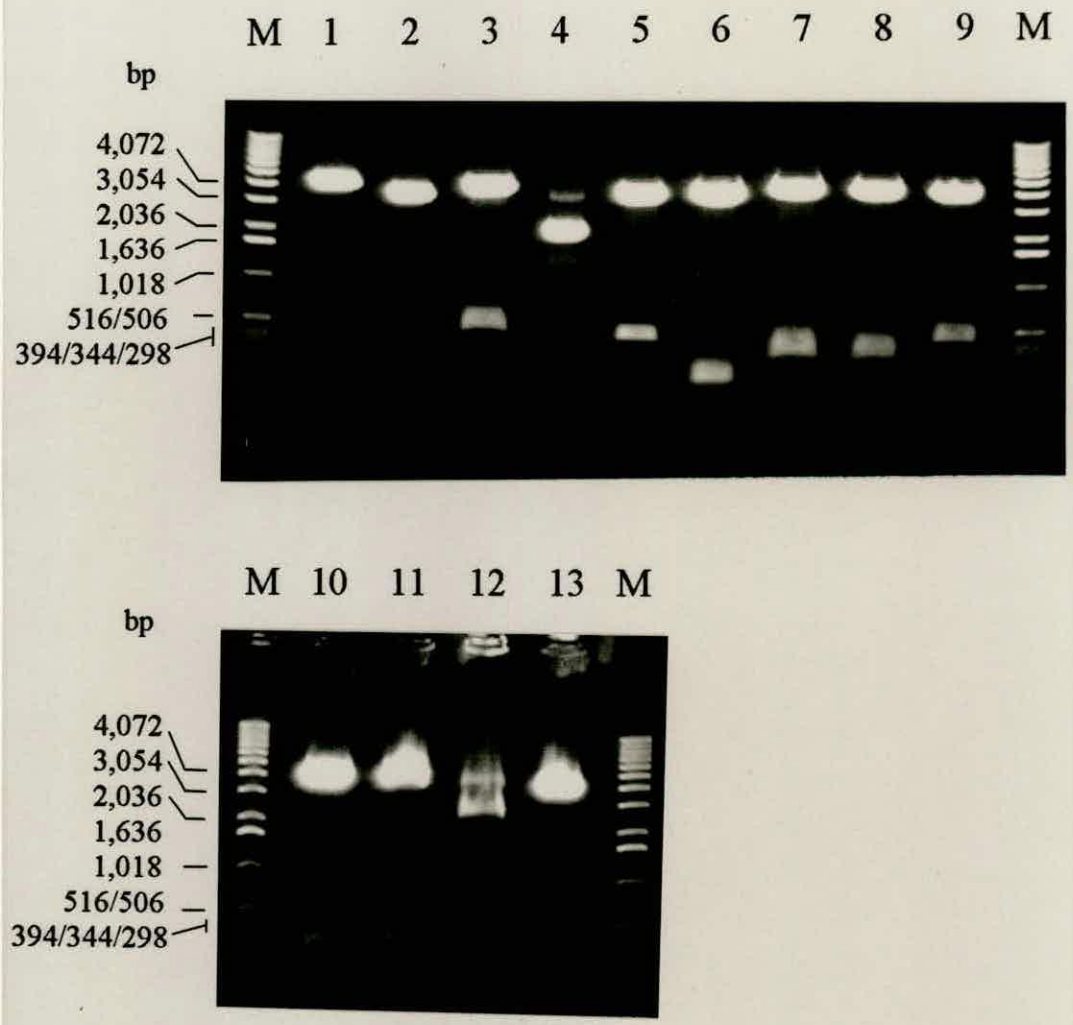
Figure 6.15

Agarose gel profile of *Eco*RI restricted plasmid DNA from recombinant clones.

The products of PCR reactions 7 and 8 were ligated into the pCR II plasmid vector. The plasmid DNA from recombinant clones was isolated and was cut with *Eco*RI to excise the insert DNA. The digests were analysed by agarose (0.8%, w/v) gel electrophoresis.

Lanes:

M - DNA markers
1&13 - *Eco*RI restricted plasmid DNA from a non-recombinant clone
2-5 - pCR II plasmid containing fragments from reaction 7
6-12 - pCR II plasmid containing fragments from reaction 8


M 1 2 3 4 5 6 7 8 9 M
bp
4,072
3,054
2,036
1,636
1,018
516/506
394/344/298
M 10 11 12 13 M
bp
4,072
3,054
2,036
1,636
1,018
516/506
394/344/298

6.3 Discussion

The work described in this chapter, aimed to characterise the cDNA fragment present in the λgt11 recombinant clone, KM19. As discussed in chapter five, KM19 harboured a cDNA insert that appeared to be expressed in the opposite orientation to lambda β-galactosidase. The derived nucleotide sequence of the insert did not encode for a complete peptide (figure 6.1). Translation from nucleotide 79 (ORF1) produced a peptide of 103 amino acids in length that was >30% identical to several types of serpin molecules, the most significant match being with LEI (figure 6.2). Upstream of this, there were stop codons in the reading frame. For the peptide to have been expressed in λgt11, there would need to have been a reading frame straight-through from one of the first three bases. However, translation of the sequence, starting from base three, gave an open reading frame for 28 amino acids (to nucleotide 86) and it may be this peptide which was expressed and recognised by the anti-adult *T. vitrinus* ES serum. The first 15 of this 28 amino acid peptide actually matches 15 amino acids in the serpin-like peptide. The two peptides would actually fall into frame with each other if there was an extra nucleotide in the sequence between bases 79 to 86, but sequencing of a DNA fragment generated by PCR amplification of *T. vitrinus* cDNA using the KM19 specific primer M0627 as well as primer H0664 (figure 6.1) confirmed the sequence shown in figure 6.1 for this region was correct and confirmed that KM19 was a genuine cDNA sequence and not a cloning anomaly generated by the chance ligation of two cDNAs into the same phage.

Attempts to amplify the 5' end of the KM19 gene, using the SL1 sense primer and an antisense primer directed towards KM19, resulted in the production of three dominant cDNA fragments, termed serp1, serp2 and serp3, of approx. 740, 540 and 500 bp in size respectively. Southern blot analysis confirmed that all three of these fragments contained sequence similar to the KM19 insert (figures 6.5-6.8). The main difference between the three serp sequences appeared to be the length of 5' untranslated nucleotide sequence between the SL1 site and a putative start codon. Serp3 was found to have a single adenosine residue between the spliced-leader sequence and the first putative start codon. Serp2 aligned exactly with serp3 apart from possessing 56 bases between the SL1 and the equivalent Met codon of serp3.

Similarly, serp1 contained an additional 144 nucleotides immediately following the SL1 sequence. Translation of serp3 gave a peptide of 168 amino acids in length which showed >30% identity to LEI and HPTI. Similar peptides were encoded for by serp2 and serp3. The last 272 bp of each serp matched bases 82-353 of the KM19 cDNA insert, however, the first 81 bp of the KM19 fragment was completely different to the equivalent regions in the serp fragments. This is the same part of the KM19 cDNA that was out of frame with the sequence encoding the KM19 serpin-like peptide. In conclusion, none of the amplified cDNA serp fragments corresponded exactly to the KM19 cDNA. Comparison of KM19 with the serp fragments and other serpin molecules suggested that if the entire sequence of KM19 encoded a serpin, the first 81 bp would form an extra region within the serpin structure. In ORF3 of KM19, the first 15 amino acids corresponds to 15 amino acids within the serpin-like peptide (which is ORF1). Alignment of KM19 with other serpins correlates this 15 amino acids to the region between helix D and helix E and includes part of strand A2 (Remold-O'Donnell, Chin and Alberts, 1992). However, at present, the author cannot explain why the first 81 bp of KM19 encodes a peptide that is out of frame with the remainder of the sequence (figure 6.1). Also, the significance of the presence of the repeat sequence and an inverted repeat region within this 81 bp is not known. The 15 amino acid stretch which is repeated in KM19 is also present (but not repeated) in the serp sequences. However, if this was the region of the KM19 cDNA that was expressed in λgt11 and selected using the anti-adult *T. vitrinus* ES serum, the antigen is not limited to the protein encoded for by the KM19 gene, but is found in the other serp fragments.

As stated above, the serp fragments mainly varied by the length of cDNA sequence between the SL1 site and the position of a putative Met start codon. There was also a single base difference between serp3 and the other two at a common position. Serp1 also possessed three other base changes when the serp sequences were compared. Though the serps differ in size, the Northern blot analysis (see below) suggested that they are derived from the same size of transcript. However, the serps may also differ in size at their 3' regions. If they do originate from the same

mRNA transcript, the different lengths in sequence from the SL1 to the putative start codon may reflect mRNA at different stages of maturation.

In the region where the serp sequences align, the few base changes may be caused by using *Taq* polymerase in the PCR reactions. Also, the base differences may be a result of worm to worm variation. In one report, amino acid diversity has been found in the 12 kDa serpin secreted by *Echinococcus granulosus* and the authors (Shepherd, Aitken and McManus, 1991) suggested that the protein may be expressed as isoforms from a polymorphic gene in response to changes in host defence mechanisms. The gene encoding a 30 kDa valosin-like protein, isolated from *T. colubriformis*, also exhibited polymorphic variation (Savin *et al.*, 1990).

The function of the SL sequence, SL1, which is *trans*-spliced to the serp sequences, has not yet been defined. Recently, work has been published describing the discovery of polycistronic operons in *C. elegans* (Spieth *et al.*, 1993; Zorio *et al.*, 1994). Spieth *et al.* (1993) proposed SL2 *trans*-splicing occurs only on mRNAs downstream from a gene located in the same orientation, therefore may be involved in cleaving polycistronic mRNAs to single mRNAs. Other proposed functions of the *trans*-spliced sequences include a method for selecting translational initiation sites and providing protection from mRNA degradation (Hirsh, 1994).

Following the discovery that KM19 differed in 81 nucleotides from the serp sequences, additional primers, M0627 and M0628, directed towards this region were designed. Though PCR amplification of adult *T. vitrinus* cDNA, using SL1 and the M0628 (KM19 antisense primer), produced a cDNA fragment in the correct size region (figure 6.12, lane 1), five fragments of similar size were found on subcloning. All these cDNAs were found to be primed at both ends with the M0628 primer. PCR amplifications using KM19 sense primers in conjunction with the dT primer were also not successful in producing a distinct product. The full length cDNA for KM19 may not actually be *trans*-spliced by SL1. Also, the dT primer requires a low annealing temperature and limits the attainable specificity of the PCR reaction.

Following this, PCR was carried out using the amplified adult *T. vitrinus* cDNA λgt11 library as the template DNA. The PCR reactions were performed using primers directed to the λgt11 sequence flanking the cloning site to primers directed

towards KM19 specific sequences. Initially, an amplification was carried out from the λgt11 arm to KM19 antisense primer, M0628. Since this KM19 primer may also anneal as a sense primer, it was possible that both the 5' and 3' regions of KM19 may be amplified, and analysis of the PCR products suggested that this may have been the case. Using this reaction as a template, amplification from the KM19 primer, M0628, to the polyA$^+$ tail with the dT primer produced a band of approx. 650 bp. This implied that M0628 was acting as a sense primer for KM19 and that the 3' region of the KM19 cDNA was being amplified. Additional amplifications of this fragment, using KM19 internal primers, H0663 and H0664, and H0663 with the polydT primer provided further evidence for the 3' end of the KM19 cDNA being present.

The PCR reactions, utilising the amplified library as template, were repeated at high stringency using the λgt11 primers and the KM19 specific primers. The reactions involving primers 514N/M0627 and 514N/M0628 produced several candidate DNA fragments that may have been derived from KM19. Subcloning of these PCR reactions produced five cDNA fragments that, upon Southern blotting, hybridised to a KM19 derived probe. However, on sequence analysis of these products revealed that none of the fragments possessed sequences similar to KM19 or any other serpin molecules. Three of the cDNAs, in fact, encoded λgt11 proteins.

Since the probe was amplified from the KM19 recombinant, as well as full length probe, small labelled fragments including the possibility of lambda DNA cross-reaction fragments, may be present, therefore, this may be part of the explanation for the positive hybridisation. This possibility could be minimised by fractionation after probe labelling by either electrophoresis/genecleaning of size exclusion chromatography. The result tends to suggest that the probe may have been contaminated. Equally, isolation of λgt11 sequences implies that the original PCR conditions used to generate these fragments were not stringent enough. Amplification of different portions of the λgt11 sequences indicated that the λgt11 primer, 514N, may not have been very specific. Also, the proportion of λgt11 DNA to KM19 cDNA present in the amplified library would have been extremely high. If the KM19 primers bound non-specifically to the λgt11 DNA, because of the amount of template, it is not surprising that amplification of the λgt11 DNA, as opposed to KM19

sequence, would be favoured. However, the initial PCR reactions carried out did indicate that there was some amplification of the 3' region of KM19.

These observations highlight some of the difficulties which may be encountered when attempting to isolate specific cDNAs by PCR. The difficulties encountered here were due, in part, to the initial restrictions imposed on primer selection by the sequence comparisons between serps 1, 2, 3 and KM19. Subsequent analysis suggested that the coding region of KM19 was not preceded by SL1 while 3' amplifications were restricted in specificity by the requirement to use low stringency conditions imposed by the use of the oligo dT primer. An alternative approach would be to screen a size selected cDNA library with KM19 and isolate putative full-length cDNAs encoding serpins. The origin of such clones (i.e. serp 1, 2, 3 or KM19) could be confirmed by 5' and 3' end sequencing.

Northern blot analysis of adult *T. vitrinus* mRNA demonstrated that the KM19 cDNA insert had a transcript size of approx. 1.2 kb (figure 6.4). This was in accord with the predicted size required to encode a serpin, based on the size of other known serpins (see table 6.1). Southern blot analysis of adult *T. vitrinus* genomic DNA resulted in a single strong band of 800 bp and a possible fainter band of 2,000 bp being recognised by KM19 in *Eco*RI restricted DNA. Also, in DNA cleaved with *Hind*III, a single faint band of approx. 4 kb hybridised to KM19. This simple hybridisation profile implied that the KM19 cDNA was probably not part of a gene from a multi-gene family.

Serpins have been isolated from the somatic preparations of several parasites including *Schistosoma haematobium* (Blanton, Licae and Aman, 1994), *B. malayi* (Alan Scott, John Hopkins University, Baltimore, USA; personal communication), *A. suum* (Hawley and Peansky, 1992), *Anisakis simplex* (Morris and Sakanair, 1994) and the rat cestode, *Taenia taeniaeformis* (Suquet, Green-Edwards and Leid, 1984). A serpin has also been identified as a major hybatid cyst fluid antigen of the dog tape worm, *E. granulosus* (Shepherd, Aitken and McManus, 1991).

The actual role of parasite serpins is currently unknown. As discussed in section 6.1.1, serpins regulate many serine proteinases that are involved in host immune mechanisms and, therefore, secretion of a serpin by *T. vitrinus* may be an important way by which the parasite modulates host immunity. *T. taeniaeformis* possesses the serpin, taeniaestatin, that inhibits host cell proteinases (Suquet, Green-Edwards and Leid, 1984), neutrophil chemotaxis (Leid, Grant and Suquet, 1987), the alternative and classical complement pathways (Leid, 1988) and interleukin-2 stimulation of T-cell proliferation (Leid *et al.*, 1986). The *S. haematobium* serpin, which is present on the surface of the adult worm, showed the most significant amino acid homology with two serpins that inhibit thrombin and, therefore, the parasite serpin may be involved in inhibition of blood coagulation (Blanton, Licate and Aman, 1994). *In vitro*, adult *T. vitrinus* ES proteinases demonstrably degrades fibrinogen and plasminogen (chapter three), proteins both involved in the blood clotting process. Secretion of a serpin by *T. vitrinus* may be another means by which the parasite interferes with blood coagulation. Also, it is possible that, like *S. haematobium* (Blanton, Licate and Aman, 1994), rather than being secreted/excreted by *T. vitrinus*, the serpin may be localised on the surface of the nematode and released into the environment by active shedding of the cuticle.

In the intestine, *T. vitrinus* is also exposed directly to serine proteinases produced from the host digestive system (for example, trypsin and chymotrypsin), neutrophil elastase and mast cell serine proteinases. Comparison of the aqueous extracts of the parasitic nematodes, *A. suum*, *A. lumbricoides* and *Toxocara canis*, with that of the free-living nematode *C. elegans*, demonstrated that the parasitic nematodes possessed greater serpin activity than *C. elegans*, leading the authors (Hawley, Martzen and Peansasky, 1994) to postulate that exposure to chymotrypsin, elastase and trypsin in the intestinal environment, for example, may stimulate the production of high levels of inhibitors by the parasitic nematodes to inactivate the proteinases.

Ascaris has been shown not to secrete proteinase inhibitors and does not inhibit hydrolytic enzymes present in the culture media. Experiments suggest that *Ascaris* spp. actually take up host proteinases and inactivate them internally (Hawley,

Martzen and Peanasky, 1994). The serpin from *A. simplex* inhibits elastase and trypsin, but not chymotrypsin (Morris and Sakanari, 1994). The hybatid cyst serpin from *E. granulosus* (Shepherd, Aitken and McManus, 1991) and the Smpi56 inhibitor of *S. mansoni* (Ghendler, Arnon and Fishelson, 1994) inhibit both elastase activity. The latter two also inhibit neutrophil chemotaxis. However, it is not known how this activity is related to the serpin mechanism.

Adult *T. vitrinus* ES serpin(s) may also regulate parasite-derived serine proteinases. In chapter three, it was demonstrated that adult *T. vitrinus* actively secretes a serine proteinase *in vitro*. The *A. simplex* serpin, in addition to inhibiting host proteinases also inhibits an *Anisakis* serine proteinase (Morris and Sakanari, 1994). An inhibitor of another class of proteinase, cysteine proteinases, has been isolated from *O. volvulus* (Luistgman *et al.*, 1992). The inhibitor, oncocystain, is present in all developmental stages of the parasite but not in the mature microfilariae. Luistgman *et al.* (1992) proposed that oncocystain may regulate a cysteine proteinase that may play a role in moulting and cuticle loss in the L3, L4 and adult stages of *O. volvulus*.

In conclusion, the results presented in this chapter form the foundation for a new direction of research in the search for host-protective antigens against *T. vitrinus*. The discovery of a putative *T. vitrinus* ES serpin has added to the knowledge of how the nematode may evade attack by the hosts immune system. Given the potential roles of a *T. vitrinus* ES serpin, it may be a molecule which stimulates potential host-protective immune responses. Future work in this area will continue to focus on isolating and expressing the full length cDNA for the KM19 prior to *in vitro* functional analysis and *in vivo* sub-unit vaccine utility. The affinity of the recombinant serpin for a panel of serine proteinases could be tested, including mast cell proteinases which are released in response to helminth infection. Stage (and species) specificity of serpin expression could be sought as well as localisation of the site of expression in the parasite by immunohistochemistry and *in situ* hybridisation. These studies would provide further information to enable the definition of *in vivo* serpin function.

Chapter seven

General Discussion

T. vitrinus is one of the principal causative nematodes of ovine PGE in Scotland. However, it is perhaps the least studied of the important ovine GI nematodes. Although the development of anthelmintic resistance by *T. vitrinus* is not an immediate threat, the emerging trend of resistance observed in other ovine GI nematodes, coupled with the growing concerns about the presence of drug residues in meat and possible environmental consequences, indicate to the need for alternative control procedures (Waller, 1993 a). In the past, the identification of parasite ES components has provided an insight into how parasites may manipulate their host environment and has targeted possible proteins for serodiagnosis, the development of vaccines or new types of anthelmintic drugs (Lightowlers and Rickard, 1988). The research described in this thesis sought to characterise components excreted and secreted by the adult *T. vitrinus* during *in vitro* culture, both at the biochemical and molecular level.

The initial part of the work involved the partial biochemical characterisation of AChE and proteinase activities present in adult *T. vitrinus* ES. A single isoform of AChE and several proteinases, including a serine and two metallo-proteinases were identified. The excretion/secretion of these enzymes *in vivo* by adult *T. vitrinus* currently is only speculative, but the potential importance of these enzymes to parasite maintenance within the host is discussed in detail in chapter three. The range of potential functions served by proteinases in particular was indicated by the variety of host proteins which were degraded by adult *T. vitrinus* ES. The preliminary studies on *T. vitrinus* L4 larvae ES proteinases suggested that proteinase secretion by *T. vitrinus* is be stage-specific. AChE and proteinases are probably only two types of many enzymes released by *T. vitrinus* during parasitic infection. Future work in this area may include analysing the ES for a wider range of enzymes and assessing the stage-specificity of the enzymes. For example, SOD has been detected in adult *T. vitrinus* ES (Knox and Jones, 1992) but the enzyme has not been biochemically characterised.

The potential involvement of parasite enzymes, such as AChE and proteinases, in the modulation of anti-parasite immune responses indicates these may be potentially useful host-protective antigens. AChE is secreted in copious quantities by many

parasitic nematodes and considerable interest has centred on this enzyme for a number of years. As discussed in the chapters one and four, the advent of recombinant DNA technology has provided an alternative approach to purifying parasite proteins.

Chapter four describes attempts to isolate adult *T. vitrinus* cDNA fragments encoding AChE using PCR and degenerate oligonucleotide primers directed at the known sequences of AChE molecules from higher eukaryotic organisms. Though this method has been successful in the isolation of other nematode enzyme genes, in this particular case the technique was fraught with difficulty. The main problem appeared to be associated with the low stringency conditions which were dictated by the uncertain homology of *T. vitrinus* AChE(s) to those from higher eukaryotes. Despite the extensive interest in nematode AChE, to date, no parasitic nematode AChE gene sequences have been published. Recently, Arpagaus *et al.* (1994) described the isolation of a gene (*ace*-1) encoding AChE (class A) from *C. elegans* using a similar approach (with two similar primers) to that described in chapter four. However, *C. elegans* possess three classes of AChE molecule (class A, B and C) encoded by three different genes (*ace*-1, *ace*-2 and *ace*-3 respectively) and the authors (Arpagaus *et al.*, 1994) suggested that *ace*-1 was distinctly different to *ace*-2 and *ace*-3. Therefore, as detailed in chapter four, *T. vitrinus* AChE may possess AChE(s) closer to *ace*-2 or *ace*-3 than *ace*-1 of *C. elegans* or may express AChEs encoded by genes quite distinct from *ace*-1, 2 or 3.

Immunoscreening of an adult *T. vitrinus* cDNA λgt11 library with serum raised against adult *T. colubriformis* sAChE resulted in the isolation of an immunopositive clone harbouring an insert encoding a myosin-like protein. The possible reasons for this are discussed in chapter five. Only a small proportion of the library was screened due to the limited amount of serum available, but more extensive library immunoscreening coupled with subsequent probing of selected immunopositives with cDNA probes encoding *C. elegans ace*-1, 2 and 3 may lead to the identification of an AChE clone. The approach involving isolation of AChE cDNA fragments was chosen because the limited amount of ES material made direct purification of the enzyme difficult. Nevertheless, it may prove necessary to try and purify AChE and attain N-terminal sequence data for the enzyme in order to

eventually isolate a suitable clone. Experiments at Moredun Research Institute (Dr. D.P.Knox; personal communication) indicated that *T. vitrinus* ES AChE does, to an extent (20%) bind to edrophonium-sepharose although the protein yield was small. If a N-terminal sequence was obtained, a specific oligonucleotide primer to the AChE could then be designed and the PCR strategy repeated.

Following the targeting of a single gene, the work in chapter five aimed to identify a wider range of ES components. Antiserum was raised to adult *T. vitrinus* ES and used to screen an adult *T. vitrinus* cDNA λgt11 library. Ten immunopositive clones were selected and these clones were expected to encode putative ES proteins. Seven of the immunopositive clones isolated harboured inserts that did not show similarity, either at the nucleotide or amino acid level, with any of the sequences presently stored in the computer databases. The unknown proteins encoded for by these clones may be of great importance to the survival of *T. vitrinus in vivo* and further characterisation of these proteins at a later date is justified. This analysis would include continued searching of databases for homologies in the light of frequent additions to the sequences available, particularly from the *C. elegans* genome sequencing project. In addition, Northern blot analysis could be employed to provide an indication of the expected size of the native ES protein and to analyse stage specificity of expression. The relevance of immunopositive clones encoding myosin and vitellogenin-like protein is debated in chapter five. Perhaps the most interesting clone isolated was KM19 which possessed a cDNA insert of 398 bp encoding a serpin-like protein, a molecule that has not previously been identified in the ES of a parasitic nematode. The implication of an ES serpin in aiding *T. vitrinus* to manipulate the host environment appeared extensive and so, warranted further study of the KM19 clone (chapter six).

To isolate the full cDNA length sequence from which KM19 originated, PCR was employed, using specific primers directed towards KM19. To amplify the 5' region, adult *T. vitrinus* cDNA was amplified using a sense primer directed towards the 22 nucleotide nematode SL1 sequence and a KM19 antisense primer, H0664. Three fragments of 728, 584 and 528 bp, designated serp1, serp2 and serp3 respectively, were amplified. Nucleotides 82-353 of KM19 matched the last 272 bp

of each of the serp fragments. However, the first 81 bp of KM19 was not represented. The main difference between the serp fragments was the length of nucleotide sequence between the SL1 primer and a putative start codon. It is not known if the three serp fragments stem from different RNA transcripts. Northern blot analysis indicated that the KM19 and the serp fragments originated from the same size of transcript and Southern blot analysis suggested that they were not part of a multi-gene complex. The 81 bp unique to KM19, possessed sequences that were repeated directly or in an inverse form further downstream and 47 bp of the 81 nucleotide fragment unique to KM19 are present in the other serp fragments in different positions. If the peptide encoded by this repeat sequence formed the epitope which was recognised by the antiserum, it is possible that the proteins encoded by the other serp fragments may also be antigenic.

The most direct approach to isolating full-length cDNAs encoding KM19 and serps 1, 2 and 3 would be to screen a size-selected adult *T. vitrinus* cDNA library with any one of the serpin encoding cDNAs isolated here and confirm the identities of the isolated recombinants as that of KM19, serps 1, 2 or 3 by 5' end sequencing. It was not possible to demonstrate conclusively that the serpin encoded by KM19 was recognised by serum antibody responses in the immune ovine host. However, ovine serum antibody responses to infection with *T. vitrinus* are very weak and the primary response to the invading parasite is expressed predominately in the intestinal mucosa via local secretary IgA responses. Future analysis of the antigenicity of KM19 would have to include seeking local antibody responses, possibly using efferent lymph as a source, and would be facilitated by subcloning KM19 into a plasmid expression vector to provide target protein in quantity. Production of the recombinant protein could facilitate the purification of native serpins by, for example, immunoaffinity chromatography. The substrate specificity of native serpins could then be assessed.

It is hoped that the work described in this thesis has helped establish a foundation for future research into the isolation of candidate antigens for vaccine development against *T. vitrinus* infection. This particular study focused on examining the adult stage of *T. vitrinus* as this stage was the most readily harvested for starting material. However, ovine GI nematodes advance through a number of different

infective stages during their parasitic life and it is possible, for example, that the serpins described are expressed at a higher level in the parasite larval stages. This could be determined by Northern blotting or reverse transcriptase PCR. In addition, helminth parasites are antigenically very complex and antigens are often expressed in a stage-specific manner requiring potentially relevant antigens to be sought in the parasitic larval stages as well. Ideally, a vaccine should target the early stages of infection to prevent pathogenesis, however, targeting of the adult stage is also valuable as it may cause a reduction in egg production, leading to a decrease in residual worm populations.

Other considerations for identifying suitable antigens should include the polymorphic variation of both the parasite and the host (Shepherd, Aitken and McManus, 1991). As mentioned in chapter six, parasites may express isoforms of proteins in response to changes in host defence mechanisms. Also, the genetic constitution of the host will be a major influence on development of resistance, in particular, the genetic repertoire of the host MHC. The class II MHC surface antigens are associated with antibody presentation and co-operation between T and B cells for lymphocyte induction. The MHC genes involved are polymorphic and result in a variable response to a particular antigen within a host population (Kennedy, 1990 and 1991).

As mentioned in the chapter one, the complexity of the nematode parasitic life-cycle makes it unlikely that vaccination against GI nematodes will produce sterile immunity (Emery, McClure and Wagland, 1993). Furthermore, given the broad specificity of anthelmintics there is little incentive to develop monovalent vaccines. For a vaccine to compete with the present drugs, the final product will probably contain either genus-specific protective antigens or antigens for each important nematode species formulated in such a way as to produce a multivalent recombinant vaccine. The incentive for vaccine production is increasing as the development of anthelmintic resistance in parasite populations increases.

The isolation of candidate host-protective antigens shows much promise with the growing knowledge of host/parasite interactions and the definition of parasite proteins of importance at this interface. However, identification of suitable antigens is

only the first step in developing a vaccine. Following this, the antigen has to be produced in quantity by recombinant DNA techniques, undergo field trials and an effective delivery system has to be established. For the development of a commercially viable recombinant vaccine against ovine GI nematodes long-term investment programmes will be required (Murray, 1987).

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